Endophytic fungi associated with cabbage in New Zealand and their potential for biological control

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Abstract

Agricultural crops such as cabbage (*Brassica oleracea* var. *capitata*) are vulnerable to a plethora of pests and diseases. Endophytic fungi are increasingly used in biological control against these pests and diseases as agricultural producers attempt to reduce their environmental footprint.

In this study, putative endophytic fungi were isolated from cabbage grown in three regions in New Zealand in order to identify isolates with potential to assist crop production. Isolation method was optimised in an exploratory study, and subsequently large scale sampling took place, where a culture-independent method was also used to detect fungi associated with cabbage. Spatial patterns in fungal communities in various plant tissues and geographical regions were investigated based on data obtained by culture-dependent and culture-independent methods.

Selected fungal isolates were tested in bioassays to determine their potential for biological control of a fungal disease caused by *Sclerotinia sclerotiorum* and an insect pest of cabbage - Diamondback moth (*Plutella xylostella*).

Over 100 species were cultured, but high-throughput sequencing suggested the number of fungal species associated with cabbage tissues could be over 300. Distinct fungal communities were found in two scales of spatial distribution - various host plant tissues and geographical regions.

Increased mortality of Diamondback moth larvae feeding on cabbage leaves treated with spore suspensions of several isolates of *Lecanicillium* sp. was observed. None of the tested isolates used as seed inoculants significantly reduced disease symptoms caused by *Sclerotinia sclerotiorum* in a bioassay on cabbage seedlings.

In this study several fungal isolates were identified as promising biological control agents of an insect pest of cabbage. However, these isolates are members of a large consortium of fungi. The patterns uncovered in the fungal communities underline the importance of understanding the microbial communities associated with agricultural crops.

Acknowledgment

I would like to thank many people and organisations, but first of all, I would like to thank my supervisory team that helped me to form ideas, question them, conceive goals and pursue them. Travis Glare, Ian Dickie, John Hampton, Mary Christey and Maria Eugenia Moran-Diez, thank you not only for that, but also for your availability and support, both professional and personal.

I am grateful for the base upon I was able to build, supported by the teachers and supervisors I was lucky to have in the past. Thank you Libor, Jana, Martin and many others. You are the giants.

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I would like to thank the New Zealand Ministry of Business, Innovation and Employment, Grasslanz and the Foundation for Arable Research for participating in Next Generation Biopesticides of which this project is a part. I would like to thank the farmers for allowing me to wander in their fields and pick cabbage plants.

Last but most importantly I would like to thank very special people; Karla, my parents and my sister with her growing family. Thank you for your love and support without which this thesis would not have happened.

[1] Note that the group of names in the section are in no particular order and have been randomly drawn using base function "sample" in R environment [99] from a pool of people that I am thankful to.

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Chapter 1

General introduction

This thesis is aligned with the Next Generation Biopesticides programme which is a joint initiative between AgResearch, the Bio-Protection Research Centre (BPRC) at Lincoln University and Plant & Food Research aimed at developing prototype biopesticides. The research is co-funded by Grasslanz Technology, an endophyte in grass commercialisation company, and the Foundation for Arable Research, a grower-good organisation.

1.1 Biological control

Growing food has always come with challenges in the form of pests, be it insects, plant pathogens, animals or competitive weeds. Several millennia of food production gave people time to develop various strategies to control agricultural pests, but since the industrial agricultural revolution in the mid 20th century, the use of chemical pesticides has become the dominant method of pest control. However, indiscriminate use of pesticides coupled with effects on human health and the environment have resulted in a need for an alternative, which can be biological control.

What it is not – conventional control

Although biocontrol has been recorded and used in agriculture for several hundred years, it was in the 20th century where it gained momentum also in scientific research [104, 144]. However, the massive use of powerful synthetic pesticides after World War II meant that biocontrol was considered an alternative to "conventional control" based on agrochemicals and cultural practices [144]. More than 160,000 tonnes of active chemicals in fungicides and insecticides were used in 2016 according to The Food and Agriculture Organisation of the United Nations (FAO) [36]. What is more, these numbers do not include herbicides or rodenticides. Hence the total pesticide use worldwide was a staggering 4,000,000 tonnes of active ingredients in pesticides in 2016. In New Zealand the estimated use of pesticides is 7.8 kg per hectare of cropland which is more than three times the worldwide average [36]. However, the advent of synthetic pesticides was soon followed by the development of resistance by both insects and microbial pests [144]. For example resistance of plant pathogens to fungicides was reviewed over three decades ago by Hartill [50].

Moreover, use of synthetic pesticides has been associated with health risks. Despite rigorous assessments of pesticide use safety, new findings show potential threats for non-target organisms and even humans [45, 76].

Biologcial control is a part of an inventory of tools that can help the needed transition towards sustainable agriculture.

What it is

Biological control (biocontrol), a term originally used to describe an introduction of exotic insects to permanently control insect pests in 1919, is now a word that has acquired various meaning depending on the pest that is controlled and the active agent [144].

For the purpose of this study, the term biocontrol will refer to purposeful use of introduced or present living organisms, other than the host plant, to reduce the pest activities or populations [53].

Invertebrate pests are generally controlled by predators, parasitoids or microbial pathogens, and the plant pathogens by antagonistic microbes [53, 135, 90, 144].

Fungi in biological control

Fungi are used extensively for biological control of other fungi, insects and plants. Entomopathogenic fungi such as *Lecanicillium*, *Metarhizium* or *Beauveria* have been used on many occasions against important insect pests such as aphids or Diamond-back moth [12, 14, 16, 20, 30, 46, 47, 121, 119, 155]. Others such as *Trichoderma*, have been successfully used protecting a range of crops from various fungal or bacterial pathogens [1, 12, 42, 58, 66, 65].

Some of the fungi used for biological control form associations with plants, such as that they reside in host plant tissues without causing any particular disease symptoms, and hereafter, these will be referred to as endophytes [12, 107, 111]. Endophytic fungi have recently gained more attention as more research is focused on identifying biological control agents in ecosystems where they are likely to operate [22].

1.2 Endophytic fungi

Fungi seem to accompany most, if not all land plants, where they colonise plant host tissues without causing apparent harm to the plant. There is evidence that such relationships were common already some 400 million years ago [107].

However, the lack of symptoms (i.e. no apparent harm nor advantage for the plant) may just be the result of our poor understanding of the interactions between partners of this association under different environmental conditions [10, 107].

To better understand the diversity of fungi that are considered endophytic, researchers try to further classify them according to the means of transmission (horizontal or vertical) and colonisation patterns and extent. According to Rodriguez et al. [107] endophytes can be further separated into clavicipitaceous (belonging to the fungal family Clavicipitaceae in the order Hypocreales) colonising a narrow

host range, and non-clavicipitaceous fungi that colonise a broad range of hosts and can be further separated into three classes based on the extent and tissue preference of colonisation and mode of transmission [107]. The clavicipitaceous fungi are often referred to as systemic and non-clavicipitaceous as non-systemic endophytes [151]. Bamisile et al. [12] further reviewed possible classifications based on the reproduction (sexual, asexual), or source of nutrition (biotrophic, necrotrophic).

Some fungi colonise the reproductive organs of the plant, "hitch- hiking" in its progeny to disperse [96, 107]. Some fungi switch to a saprotrophic lifestyle upon host plant senescence, while others may be latent pathogens waiting for their turn [67, 107, 98]. Whatever the strategy of an endophytic fungus is, the line between displaying mutualistic or antagonistic interactions is a fine one. It may only take one gene to turn the outcome; for example, wild type strain of the endophytic fungus *Epichloë festucae* on grass *Lolium arundinaceum*, acted as a mutualist while the mutant (lacking the *so* gene involved in anastomosis) killed the plant [25].

Whether the symbiosis is mutualistic, neutral or even pathogenic seems to depend also on the environmental factors such as availability of nutrients and stress from herbivory, and even nutrient flux in the host plant [35].

Although we are far from understanding the role of often complex communities of endophytic fungi, there is a growing body of research on how to harness the mutualistic interactions between endophytic fungi and plants.

Several studies identified endophytic fungi which alleviated stress from abiotic factors. For example *Piriformospora indica*, an endophyte from the Sebanicaceae family, increased drought tolerance in Chinese cabbage through up-regulation of drought related genes in the plants [127]. This fungus also boosted growth and phosphorus transfer in *Arabidopsis thaliana* [115]. Another study showed *Fusarium* endophytes to confer tolerance against heat and salt stress to their grass hosts [106] Many other studies have reported endophytic fungi to improve plant growth and vigour [12, 151].

The ability of endophytic fungi to protect their hosts from biotic factors such as herbivory or plant pathogens has received substantial interest as well.

1.2.1 Endophytes for biological control of insect pests and pathogens

As mentioned above, fungi such as *Lecanicillium*, *Metarhizium*, *Beauveria* or *Trichoderma* are used in biological control of insect pests or plant pathogens quite extensively. All of the aforementioned are to some extent endophytic.

The mode of action, how the host plant protection is achieved, varies according to the controlled organism. Fungi that control plant pathogens use strategies such as competition for space within host plant tissues, antibiosis by producing toxic metabolites, mycoparasitism, induced systemic resistance of the host plant and they can even increase plant vigour as a mean of overcoming the damaging effects of the pathogen [12]. Similarly endophytic fungi involved in control of insect pests have been reported to employ methods such as production of toxic metabolites, infecting insect tissues or producing metabolites that deter feeding or delay development [12].

Despite years of research, the range of available products and fungal species used

in biological control is still somewhat limited. Many available commercial products target a single major pest, which limits their potential [42]. Therefore pursuit for biological control agents continues to drive the search for endophytic fungi.

1.2.2 Methods of detection

For the purpose of testing fungal endophytes experimentally, one must isolate and culture them first.

Isolation of endophytic fungi from plant tissue can be approached by fragment plating. Fragment plating is a widely used technique where fragments of usually surface sterilised plant tissue are placed on a growth medium supporting growth of fungi. Different types of media may favour different subsets of fungi. As rapidly growing fungi may obscure the presence of more slowly growing species, weak media used for initial isolations may prevent overgrowth of the latter. Selective growth inhibitors and antibiotics can be used to retard growth of particular groups, suppress bacteria, and enable detection of less aggressive fungi [125].

However, culturable isolates will probably represent only a subset of the whole endophytic community, since non-culturable isolates will be missed [125]. Furthermore, the large-scale effort required for culturing is usually beyond the scope of most research projects. Therefore, other approaches should be considered to reliably describe endophytic communities and better understand factors shaping them.

Culture-independent methods are usually based on DNA sequences allowing discrimination between fungal taxa. DNA barcoding is a widely used, and well established tool for fungi, where standardised short sequences (500- to 800-bp) are sequenced to identify species using primers that are applicable for the broadest possible taxonomic group. The nuclear ribosomal internal transcribed spacer (ITS) region serves as a universal DNA barcode marker for most fungi [112].

High-throughput sequencing (HTS) methods such as 454 pyrosequencing or Illumina outperform earlier culture-independent approaches (including terminal restriction fragment length polymorphism, cloning or denaturing gradient gel electrophoresis) in terms of resolution and magnitude. They enable qualitative and semi-quantitative description of the fungi that are present in a sample (ranging from plant tissues to plant communities in fields or forests), thus offering new insights into the often complex endophytic fungal community ecology [75].

1.3 Cabbage

Cabbage (*Brassica oleracea* var. capitata) was chosen as a model host plant in this project since it is an important crop plant in New Zealand and worldwide, sharing many pests with its no less important relatives from the *Brassica* genus.

1.3.1 Cabbage is a *Brassica*

Brassicaceae is a diverse family of 350 genera and more than 3000 species. One genus, *Brassica*, encompasses important food crops such as cabbage, broccoli, mustard, forage crops such as kale, swede or turnip and oilseed crops represented by oilseed rape [11].

The phylogeny of commercial *Brassica* species can be best described by the "Triangle of U" (Fig.1.1) where three diploid *Brassica* species *B. rapa* (syn. *B. campestris*), *B. nigra* and *B. oleracea* have hybridised in all possible combinations to produce three allotetraploid species *B. juncea*, *B. napus* and *B. carinata* [88, 124]. Tetraploid forms of *B. rapa* and *B. oleracea* have also been developed.

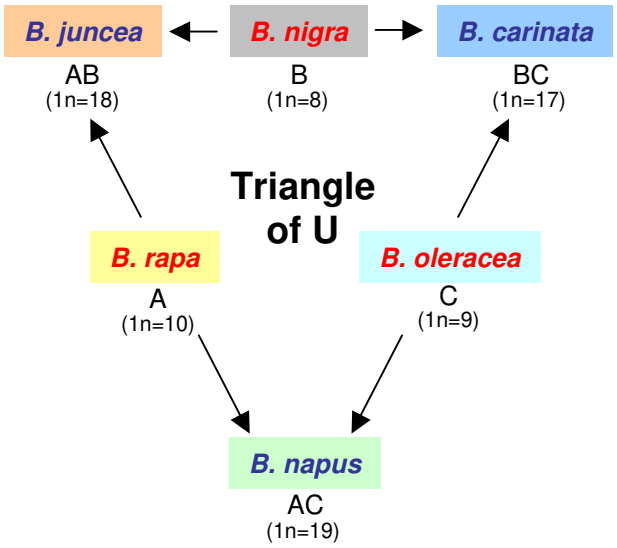


Figure 1.1: The relationship between the *Brasssica* species, known as the "Triangle of U". Diploid species are indicated by red font, allotetraploid species by blue. Combinations of genomes are indicated below the species names, and the number of chromosomes in the haploid genomes is indicated in parenthesis (taken from Østergaard and King [88]).

1.3.2 Production of cabbage and other *Brassica* crops

The Food and Agricultural organisation of the United Nations estimated that vegetable *Brassica* were grown on more than 3.9 million hectares in 2017 (cauliflower and broccoli on 1.4, and cabbage and other brassicas on 2.5 million hectares). The production was more than 97 million tonnes (with cabbage and other brassicas responsible for 71 and cauliflower and broccoli for almost 26 million tonnes produced) [36].

In New Zealand

According to Statistics New Zealand Tatauranga Aotearoa, edible cultivars of *Brassica oleracea* were cultivated on 3622 hectares (cabbage on 804, broccoli on 2,082 and cauliflower on 746) in the year 2017 in New Zealand [123]. *Brassica* species are important for seed production in New Zealand and, although vegetable brassicas represented less than 1% of total seed production, together with forage brassicas such as rape and kale and oilseed rape, they constituded more than 10% of total seed production in New Zealand in 2011 [49].

1.3.3 Pests and diseases

Cabbage just like many other *Brassica* crops is under threat from several pests. Two species representing an insect pest (Diamondback moth – *Plutella xylostella*) and a fungal plant pathogen (Sclerotinia – *Sclerotinia sclerotiorum*) were chosen for this study, because not only do they exhibit a cosmopolitan distribution but also they attack a wide range of crops. Furthermore, both these pests have devoloped resistance to "conventional" control methods. A more detailed introduction on *Sclerotinia sclerotiorum* and Diamondback moth is given in Chapter 4.1 and Chapter 5.1 respectively.

1.3.4 Why cabbage?

The bulk of work investigating endophytic associations, pests and their interactions has been done on oilseed rape (*B. napus*). Oilseed rape is a hybrid of *B. oleracea* and *B. rapa*. Research on *B. oleracea* lags behind research on *B. napus*. Furthermore broccoli, cabbage, cauliflower and kale are all forms of *B. oleracea* and of economic importance. Hence *B. oleracea* was selected as the focal species for this project. Cabbage, as a representative of *B. oleracea*, was chosen because of the growing properties and it already had established systems for study at the Bio-Protection Research Centre.

1.4 Aims

The overall aim of this study was to isolate and identify fungi associated with cabbage grown in New Zealand; and examine the potential of isolated fungal strains for biological control against insect or fungal pests and describe patterns in fungal communities detected in cabbage.

Three objectives were set up to achieve this aim:

- firstly isolation of endophytic fungi from cabbage was optimised, comparing two methods of fragment plating
- secondly, the optimised isolation method was used to recover fungi, and simultaneously, culture-independent detection method (high-throughput sequencing) was used to survey the communities of fungi associated with cabbage plants across New Zealand

- and finally, collected fungal isolates were evaluated in a series of bioassays for their potential to control a fungal pathogen *Sclerotinia* or insect pest Diamondback moth (DBM).

The objectives and outputs are summarised in Figure 1.2

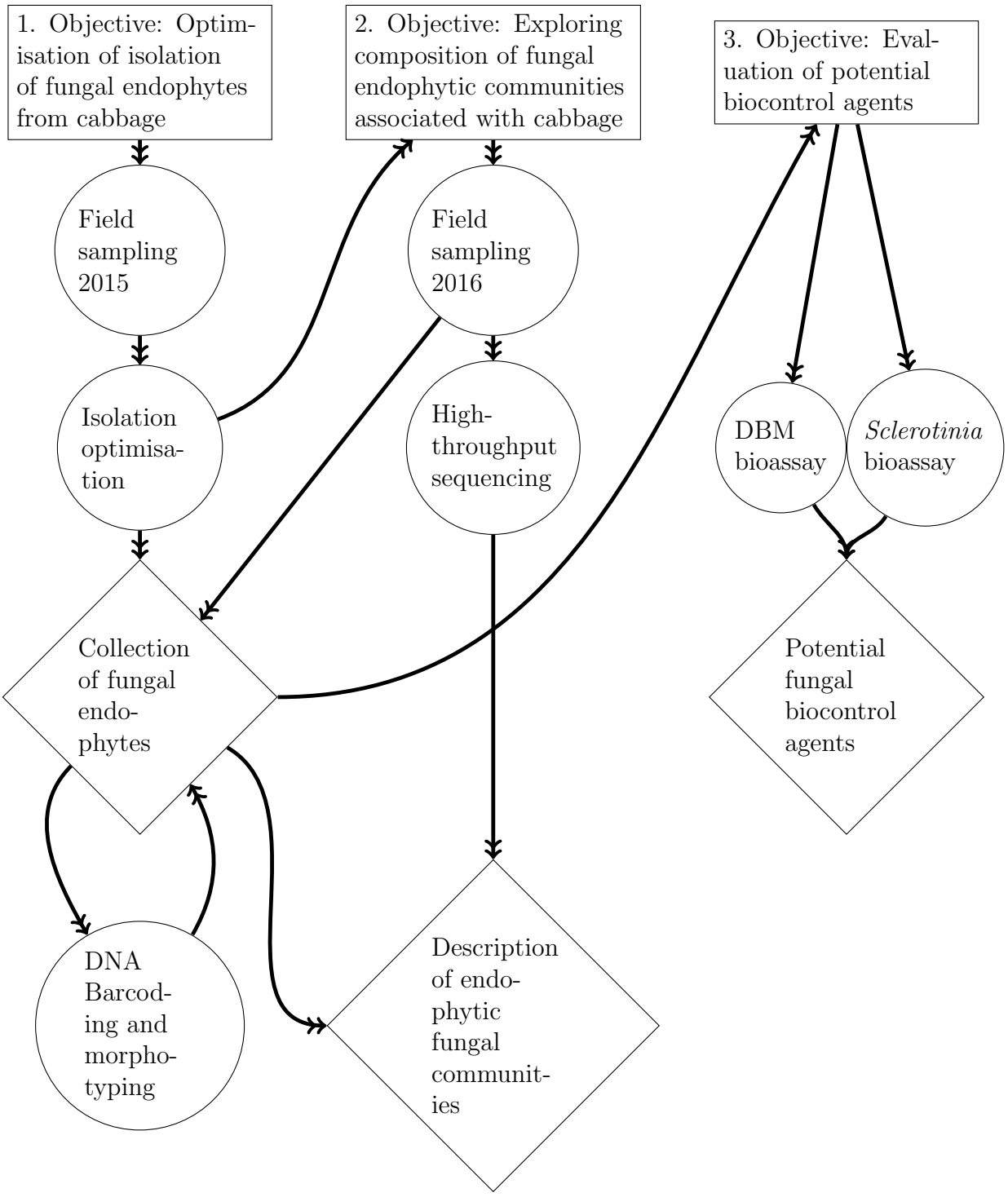


Figure 1.2: Workflow of this thesis. Objectives are summarised in the upper row of rectangles, means to achieve them are described in circles, while diamonds represent the outputs of the objectives. Arrows represent interactions.

Chapter 2

Optimisation of isolation of fungal endophytes from cabbage

2.1 Introduction

Associations between fungi and plants are ubiquitous in natural plant communities. Endophytic associations, where a fungus inhabits the inner tissues of a plant host, are receiving more and more attention, as many of the endophytes can be mutualistic or used to improve the performance of the host plant [107].

Many studies have focused on isolating endophytic fungi from symptomless plants in order to find fungi that could be used in biological control. In these studies several different approaches have been used, each with its advantages and disadvantages.

A widely used technique to isolate endophytes from plant tissues is fragment plating, where surface sterilised fragments of plant tissue are placed in a Petri dish with a medium capable of sustaining fungi that emerge from within the fragments. Sometimes selective culture media and conditions are used in order to target specific fungal taxa, however at other times researchers want to describe the diversity of endophytic fungi associated with the host plant.

Different types of media favour different subsets of fungi. Weak media with low nutrient content are preferred for initial isolations, as they can prevent rapidly growing fungi from obscuring the more slowly growing species. Selective growth inhibitors and antibiotics such as Rose Bengal can be used to retard growth of particular groups of fungi, suppress bacteria, and enable detection of less aggressive fungi [125].

Using selective media for isolation of specific fungal taxa has a long history, and has been widely implemented to target fungal endophytes such as *Trichoderma* [34, 93] or *Beauveria* [101] and many others. The selective media reflect nutritional demand of the targeted fungus and are often amended with substances that slow down growth of possible competing microorganisms [125, 63].

On the other hand, where the highest diversity of recovered fungi is desired, non-selective media able to accommodate many fungal species are preferred. Again, these can be amended by bacteriostatic or bacteriocidal agents to suppress bacterial growth [125, 89].

However, there is a bias in cultivation-dependent studies, where fast-growing, ubiquitous species are favoured, while less frequent species with specialised requirements may remain under-represented or undiscovered [140].

The current study attempted for the first time to gain insight into the diversity of endophytic fungi associated with cabbage plants. In doing so, two approaches to fungal isolation were compared; fragment plating on a single medium in a regular round Petri dish and fragment plating in a multiwell dish with several media differing in bulk nutrient content and the presence of Rose Bengal as a growth inhibitor. Not only were different types of media expected to target different subsets of culturable fungi from sampled plants, fragment plating in multiwell plates was expected to prevent cross contamination by fast-growing fungi, therefore increasing the chances of isolation of slower growing species or strains [125].

2.2 Materials and methods

Ten healthy cabbage plants were collected in March 2015. Five plants were sampled from an organic farm in the Future Farming Centre, Lincoln University and five plants from a field under conventional horticultural management near Rangiora, Canterbury.

Fragment preparation

The plants were washed in tap water to remove soil particles on the roots and the basal stems. The segments of leaves, stems and roots were surface sterilised following the procedures used in the BPRC [79] and similar to Zhang et al. [153]. Briefly samples were rinsed in 0.01% Triton X for 2 min, then in 2.5% sodium hypochlorite (v/v) for 5 min, then in 70% ethanol (v/v) for 1 min, and finally rinsed in sterile water 3 times (1 min each). Afterwards the ends or edges of segments were cut off and the remaining parts cut to obtain 5 mm fragments which were plated in dishes with cultivation media. In total, 990 plant fragments were prepared from 10 plants.

Single medium plating

Petri dishes (9 cm in diameter) containing potato dextrose agar (PDA; Difco, BD, USA) amended with chloramphenicol (Sigma-Aldrich) (100 mg/l to suppress bacterial growth) were prepared. Fifteen segments were prepared for each type of plant tissue (roots, stems and leaves) and placed in Petri dishes, five per dish in three replications (Figure 2.1). In total 450 fragments were plated on PDA.

Multiple media plating

Three different types of media were used for multiple media plating, namely PDA, Sabouraud dextrose agar (SDA; Difco, BD, USA) and Aspergillus minimal medium (AMM) prepared according to Hill and Käfer [54]. Key contents of the of the media in g/l are presented in Table 2.1 below.

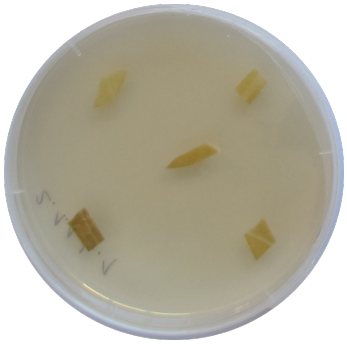


Figure 2.1: Single Petri dish with Potato dextrose agar and five fragments of cabbage tissue.

	Dextrose	Peptone	Potato starch
AMM	10	0	0
PDA	20	0	4
SDA	40	10	0

Table 2.1: Key contents of potato dextrose agar (PDA), Sabouraud dextrose agar (SDA) and Aspergillus minimal medium (AMM) in g/l.

Chloramphenicol (100 mg/l) was added to all the media to suppress bacterial growth. Each medium was used with and without the addition of Rose Bengal (100 mg/l) thus gaining 6 different types of media, one in each well (4 cm in diameter) of a multiwell plate. Eighteen segments were prepared for each type of plant tissue (roots, stems and leaves) and placed in multiwell plates, six per plate in 3 replications (Figure 2.2). In total 540 fragments were plated in plates with multiple media.

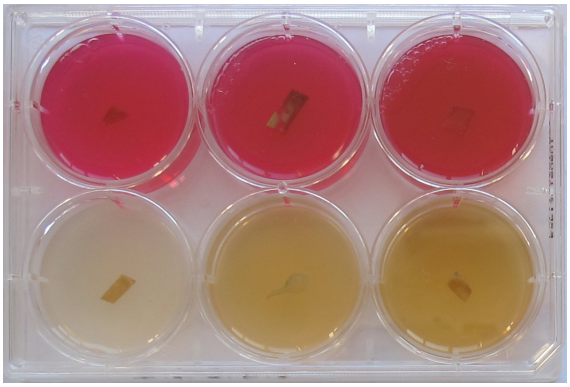


Figure 2.2: Multiwell plate with three different media in columns ammended with Rose Bengal (top row) with six cabbage tissue fragments.

Incubation

The dishes with plant tissues were incubated in the dark at 20 °C and examined daily for fungal growth for up to 2 months. The fungal colonies were individu-

ally transferred to new PDA dishes, one colony per dish, and incubated at 20 °C. The resulting fungal cultures were purified by single-spore or single-hypha-tip isolation. Finally, the pure cultures were stored in microtubes in 20% glycerol (v/v) refrigerated at 4 °C and simultaneously at −80 °C.

Morphotyping

In total, 990 plant fragments were plated and isolated fungal strains were classified into morphotypes based on macro- and microscopic characteristics such as the colour and texture of the mycelium, spore size and arrangement. Representative isolates from each plant part were identified by sequencing an internal transcribed spacer (ITS) region of ribosomal DNA as described below.

DNA extraction

This was done using Chelex® 100 Resin [145]. Tissue samples were ground in a microtube in 500 μl of 5%(w/v) Chelex® 100 buffer. After brief but thorough vortexing, samples were incubated in boiling water for 12 minutes, cooled down to room temperature and centrifuged at 13000 rpm for 12 minutes. 200 μl of the supernatant was stored refrigerated and used for PCR.

PCR and DNA sequence manipulation

The whole ITS region of each isolate was amplified using the primers

ITS1F (3' CTTGGTCATTTAGAGGAAGTAA 5') [39]

ITS4 (3' TCCTCCGCTTATTGATATGC 5') [147].

The ITS sequences were assigned to the most closely-related fungal species in the UNITE (accessed on 27.12.2016) and NCBI RefSeq Targeted Loci Project (accession number PRJNA177353, accessed on 27.12.2016) databases using the BLAST [4] program, with consideration of observed colony and spore morphology to confirm the taxonomic status of the investigated fungal isolate. Priority was given to matches from the UNITE database unless the best match was an unidentified taxon, in which case a match from the NCBI database was used.

Statistical comparison of recovery rate

The average number of isolates/species was compared by normalising the sampling effort and comparing the likelihood of retrieving a fungal isolate or a species for each plate. A rarefaction analysis was conducted to evaluate the likelihood of obtaining more species by any of the methods. Briefly, "n" (1 to 494) random fragments were repeatedly (1000) selected from both methods. The average number of species and estimated species richness was compared by one way ANOVA for each method. The likelihood of obtaining the observed number of isolates/species from the number of fragments used within each isolation method was also compared by Fisher exact test [99].

A species-accumulation curve as well as predicted species richness were based on species abundance for both isolation approaches and were calculated using the function "specpool" in "vegan" package [87].

The distribution of shared or unique fungal taxa between methods, plant tissue or fields sampled was displayed in Venn diagrams. To test whether such distribution was expected by chance, a null expectation model was based on the obtained data using the "permatfull" function in "vegan" package [87]. Isolate numbers for each species and each tissue type and isolation method were kept constant in the simulated community matrices.

Differences between fungal community composition based on isolation approaches were tested on community data converted into Jaccard distance matrices. The results were visualised in non-linear multidimensional scaling graphs and tested for significance using permutational MANOVA test with the "adonis" function in "vegan" package [87]. All statistical calculations were performed in R [99]. A working example of R code is provided in a separate file deposited at Lincoln University Data Centre (Appendix B - R scripts. doi: 10.25400/lincolnuninz.9122549).

2.3 Results

2.3.1 Detected fungal taxa and their distribution

In this study 488 fungal isolates were recovered from cabbage tissues and these represent a total of 68 fungal species (Figure 2.3). All isolates recovered along with additional information are summarised in a separate file deposited at Lincoln University Data Centre (Appendix C - Appendix C Endophytic fungi from cabbage in New Zealand 2015. doi: 10.25400/lincolnuninz.9122513).

Most of the isolated species were recovered from stems closely followed by roots (Figure 2.4). There were more species unique to roots than expected based on the null model. Eleven species were isolated from all plant parts which was less than expected, however these eleven species were among the most abundant fungi, accounting for 74.4% of all isolations.

Similarly, roughly a third of the species were recovered from both fields sampled, however, these represented 84.2% of all isolations (Figure 2.5).

2.3.2 Which fragment plating method is more efficient?

The number of fungal isolates, recovered species and expected species richness was higher under the same sampling effort for isolation on multiwell plates with different media compared to isolation using a single medium (Figure 2.6).

Out of 495 tissue fragments in multiwell plates with different media, 328 isolates falling into 59 species were recovered, whereas the regular Petri dish and a single medium approach recovered 160 isolates in 33 species from 450 cabbage tissue fragments. The estimated species richness based on data from all samples was 144.5. However 43 and 107 species were expected to be found by the single medium and multiwell plate with different media respectively.

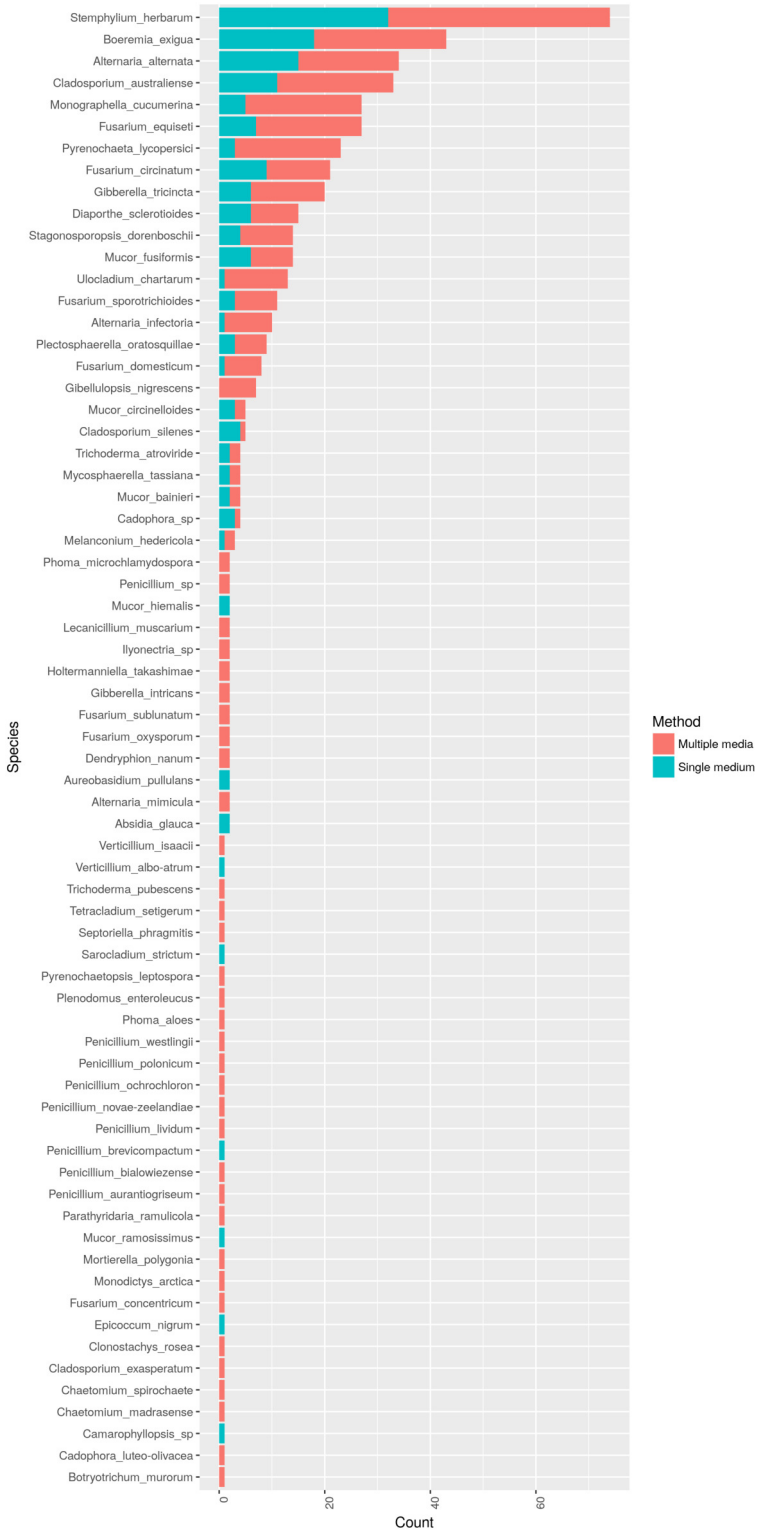
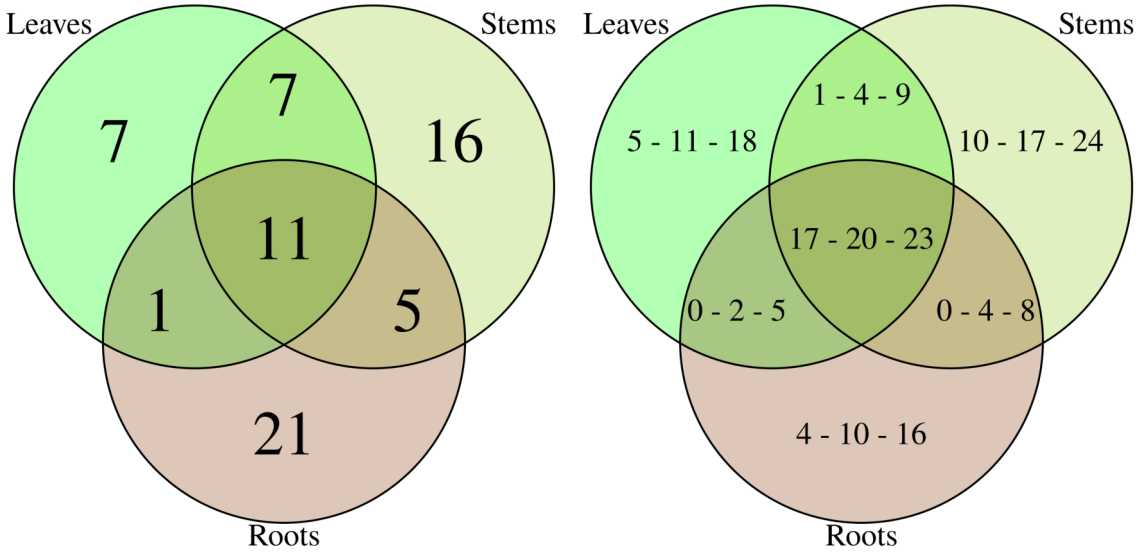


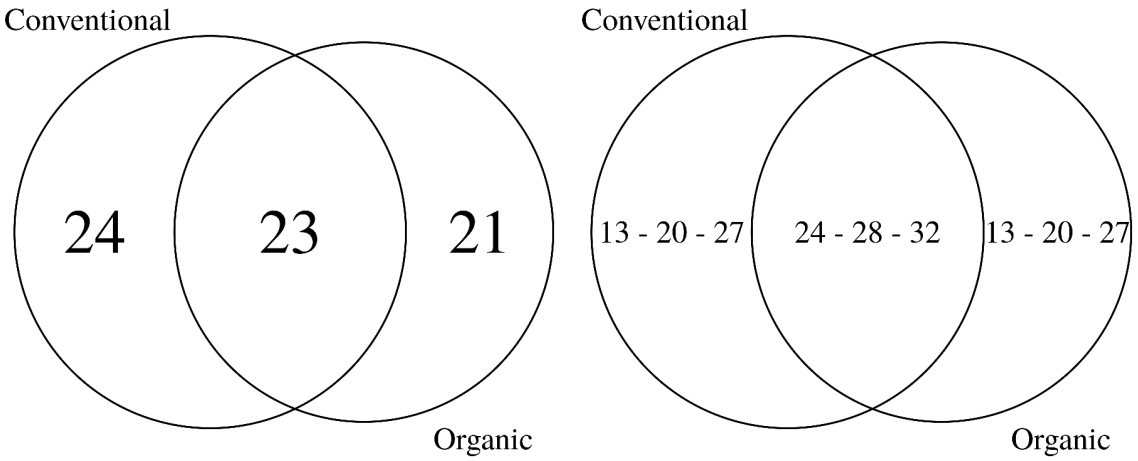
Figure 2.3: Species ranked by the total number of strains isolated by single medium isolation (blue) and multiwell isolation with different media (red).

Twenty four fungal species belonging to 17 genera were isolated by both methods and these accounted for 92.5% and 84.6% of all isolations for single medium and



(a) Observed (b) Expected

Figure 2.4: Venn diagram showing (a) observed and (b) expected number of isolated species from each type of plant tissue sampled. Expected numbers were based on random sampling of 9999 iterations with 95% confidence intervals shown (lower confidence interval - mean - upper confidence interval).



(a) Observed (b) Expected

Figure 2.5: Venn diagram showing (a) observed and (b) expected number of isolated species from each field sampled. Expected numbers were based on random sampling of 9999 iterations with 95% confidence intervals shown (lower confidence interval - mean - upper confidence interval).

multiwell plating respectively (Figure 2.7). The remaining species recovered only by one of the two approaches were isolated on average from 1.5 fragments.

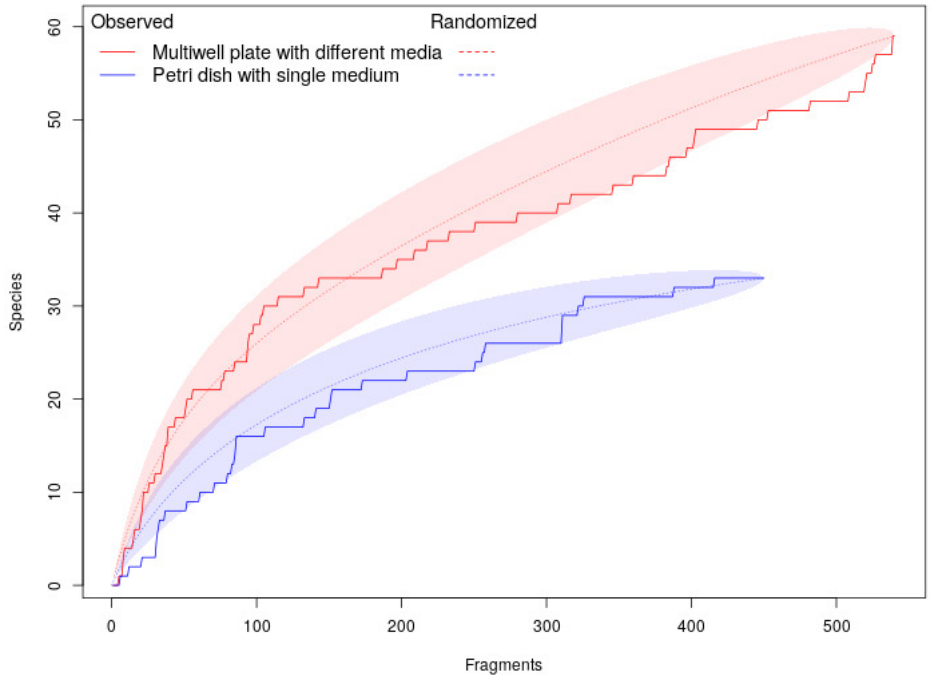
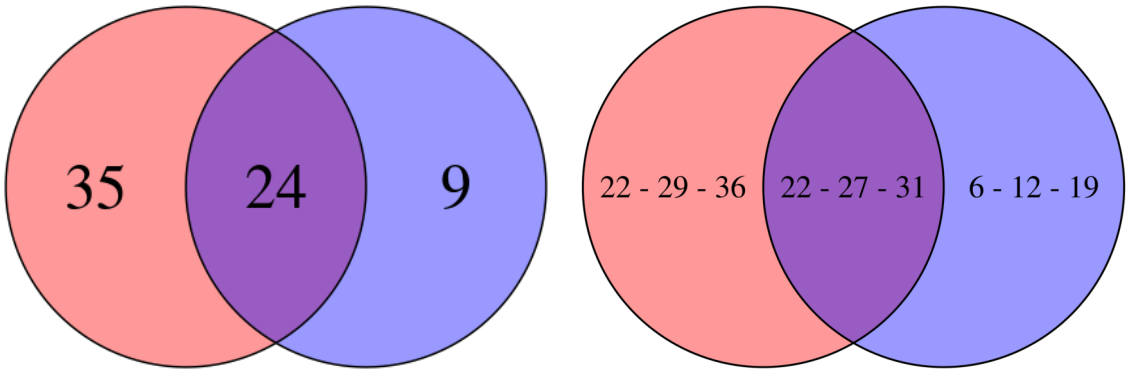


Figure 2.6: Species-accumulation curves plotted as obtained (solid line) and based on randomisation of samples (dashed line) with standard deviation (shaded area) are shown for single medium isolation (blue) and multiwell isolation with different media (red).

Different communities of fungal taxa were isolated with the respective isolation approaches, although variability explained by choice of isolation method was only 8% at p=0.029 (Figure 2.8).

The subsets of fungi isolated on multiwell plates were not strongly influenced by the choice of cultivation medium or the use of Rose Bengal (Figure 2.9).



(a) Observed (b) Expected

Figure 2.7: Observed (a) and expected (b) expected number of fungal species as recovered by single medium isolation (blue) and multiwell isolation with different media (red). The overlap (purple) represents species isolated by both approaches. Expected numbers were based on random sampling of 9999 iterations with 95% confidence intervals shown (lower confidence interval - mean - upper confidence interval).

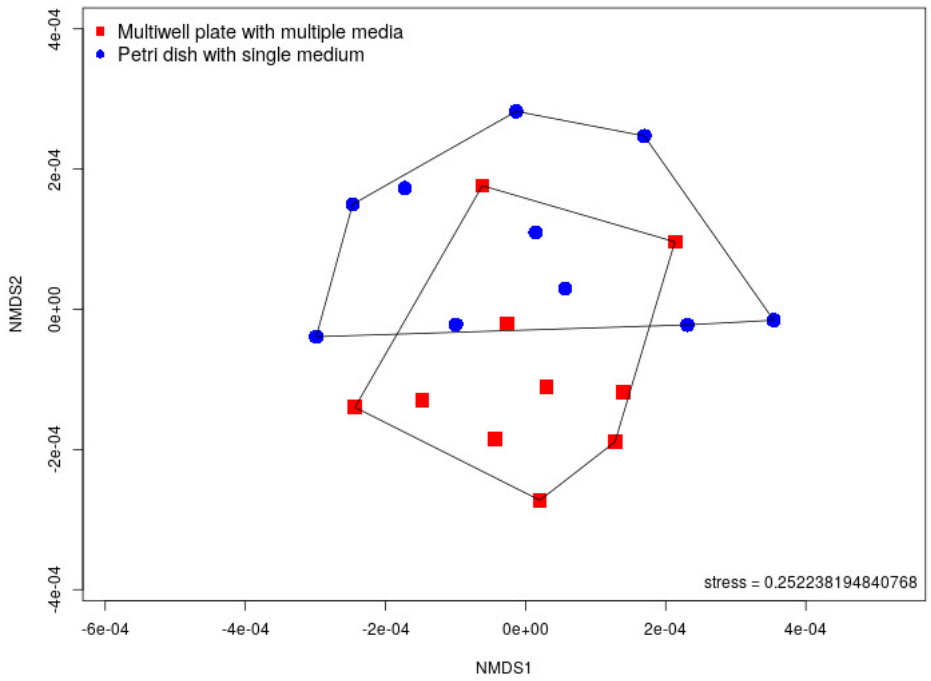


Figure 2.8: Multidimensional scaling of fungal communities as recovered by single medium isolation (blue square) and multiwell isolation with different media (red circle).

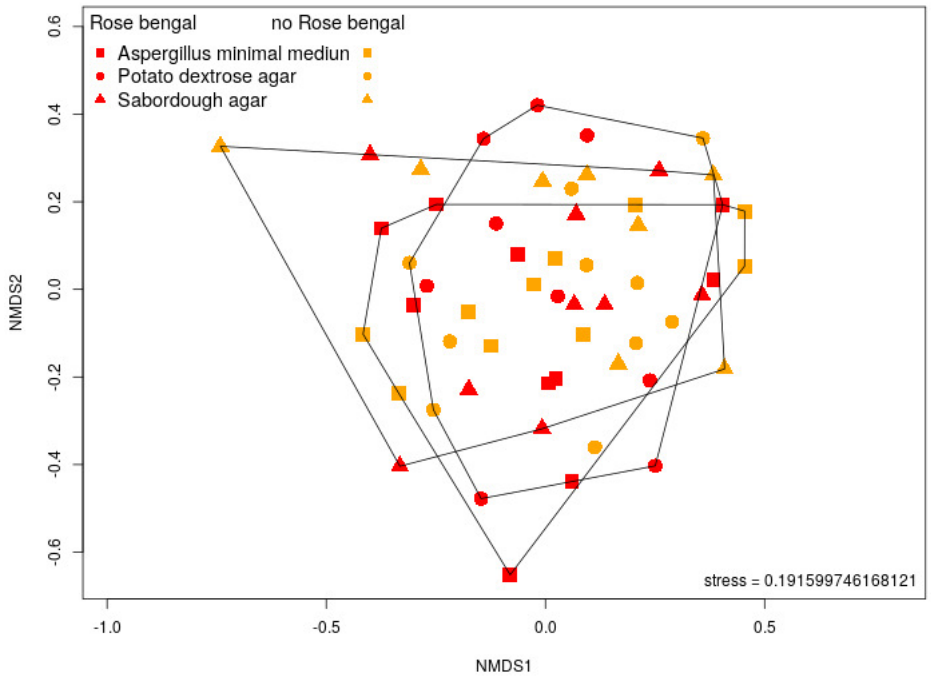


Figure 2.9: Multidimensioal scaling of fungal communities as recovered by different media: Aspergillus minimal medium (square), Potato dextrose agar (circle) and Sabordough agar (triangle) with (red) and without (orange) Rose Bengal in multiwell plates.

2.4 Discussion

2.4.1 Detected fungal taxa and their distribution

Fungi isolated in this study belong to fungal genera not uncommon in literature on endophytes. Fungi such as *Alternaria*, *Chaetomium*, *Cladosporium*, *Fusarium*, *Mucor* and *Trichoderma*, were not only endophytic, but could also display beneficial activity towards their *Brassica* host plants [22].

Based on classification of potential endophytes by Rodriguez et al. [107], fungi found throughout host plants and ones restricted to certain types of tissue were expected to be isolated in this study. On average, twenty fungal taxa were expected to be shared across all types of plant tissue, however, only eleven were isolated. On the other hand, 44 species were isolated only from one type of tissue, with over-representation of fungi associated with roots (Figure 2.4). Schulz and Boyle [113] claimed that endophytic colonisation is usually more extensive in roots than in shoots or leaves, however, more species were isolated from stems than any other tissue in current study. Nevertheless, relatively high degree of tissue specificity or preference could be driven by fungi isolated from roots. Current study focused on comparing isolation methods rather than describing fungal communities associated with cabbage. Only two field sites differing in horticultural practices were sampled, hence conclusions about patterns of endophytic fungal communities between horticultural practices cannot be drawn from this study. What is interesting though, is that the twenty three species recovered from both fields accounted for more than three quarters of all isolations and suggest an abundant (or easy to isolate) subset of fungi associated with cabbage shared across fields. These results align with a study on endophytes in oilseed rape, where *Alternaria alternata* represented 12.4% of all isolations and several other species were isolated relatively frequently, while the majority of isolated fungal species were isolated only once [153].

That is why more robust and detailed sampling was conducted in a consequent study (as described in Chapter 3), to address spatial distribution of fungal taxa in various scales.

2.4.2 Comparing methods

Fragment plating in multiwell dishes yielded not only more isolates recovered but also more species than plating in regular Petri dishes. This is most likely due to confined growth space for each fragment preventing overgrowth of slower growing fungi by those with vigorous growth.

The data from the multiwell plates (2.9) suggest that the choice of growth media did not influence the spectrum of isolated fungi, however *Gibellulopsis nigrescens* was isolated seven times, six of which were on Aspergillus minimal medium, which is the medium with the lowest nutrient content. This species was not isolated on regular Petri dishes.

If enough sampling effort is made and all the species present in an assemblage are detected, the species-accumulation curve should reach a plateau. In this study species-accumulation curves for both isolation methods did not reach that stage

(Figure 2.6), which was also supported by estimated species richness expected to be higher than observed in both cases.

Nevertheless, the distribution of fungal taxa recovered by any of the two methods are not beyond random expectations, hence, the recovery rates in both datasets can be compared with reasonable confidence. Furthermore, it can be concluded that more fungal taxa could be recovered from cabbages in New Zealand, if the sampling effort was higher.

Chapter 3

Fungal communites

3.1 Introduction

3.1.1 Fungal communities associated with plants and their patterns

Plants can no longer be considered standalone entities because of the wide diversity of microorganisms which they harbour. Endophytic fungi can be found in tissues of all land plants, and the composition of the fungal communities within and between plants may be influenced by factors such as plant host, its genotype, tissue or environmental factors such as soil properties, climate or interactions with other organisms associated with the host plant [125, 107]. There is growing evidence that specific fungal communities inhabit specific plant species or genotypes. Endophytic fungi differ in their host preference (i.e. some fungi have a narrow host range while others are able to establish symbiosis with a broad range of plant taxa). This means that some plants will share generalist fungal endophytes while the fungal communities will differ in specialist fungal endophytes [125, 107].

Some endophytic fungi will colonise the whole plant including the seeds; these are often referred to as systemic [125]. However, some fungi are restricted to certain host tissues, or part of the plant. A well studied example is mycorrhizal symbiosis where the fungus is associated only with the roots. Non-systemic, non-mycorrhizal endophytes can display patterns in host plant colonisation. Numerous studies have identified distinct fungal communities in different plant parts. In a review by Rodriguez et al. [107], the authors divide endophytic fungi in classes based on whether they colonise shoots or roots of host plants and whether the colonisation is extensive or scarce.

Environmental conditions can also influence the composition of the endophytic fungal community hosted by a plant. Properties of soil in which the host plant is growing are a well studied factor influencing structure of fungal communities associated with plants. Agricultural management was shown to affect the endophytic communities of grapevine (*Vitis vinifera*), maize (*Zea mays*) and several grasses, in recent studies [91, 114, 148].

Endophytic fungi form complex assemblages in their host plants. These assemblages are likely non-random and the distribution of species is affected by plant

species, their tissue type and environmental factors [107, 125].

3.1.2 Isolation and detection methods

The pursuit of beneficial fungal endophytes associated with crops has its limits. The chosen isolation method and sampling effort can greatly affect the fungal communities that are isolated in a given host plant system. There is a theoretical possibility of isolating all culturable fungi from a system, however, increasing sampling effort or using different isolation methods might not always be feasible, and what is more, non-culturable fungi will be missed [125].

Culture-independent, DNA sequence–based detection methods are revolutionising the investigations of fungal diversity. Metabarcoding is one of these methods and involves PCR to amplify target DNA regions extracted directly from mixed environmental samples. Metabarcoding has been used in many studies of fungal diversity to describe mycorrhizal symbionts [60, 137], foliar endophytic communities [64], soil fungal communities [86, 132] or even fungi associated with strawberries under different management practices [2].

Although metabarcoding is a promising tool in fungal diversity studies, it does not come without caveats. Many of the studies investigating fungal endophytic communities use the internal transcribed spacer (ITS) region of the fungal nucleic DNA as a barcoding region. There is a trade-off in primer selection for initial PCR; different primers are biased towards different fungal lineages and there is also a possibility of amplification of plant DNA since the mixed DNA samples necessarily contain significant amounts of it [17, 136]. Furthermore, based on the PCR conditions, DNA of some fungal lineages may be preferentially amplified due to lineage dependent ITS length variations [17, 133]. Finally, HTS is prone to biases during the sequencing process, resulting in a problematic relationship between read counts and real biological differences [134]. Nevertheless, with an awareness of the limitations of chosen markers, the primers to amplify them and bioinformatic and methodological challenges, HTS studies can provide an in-depth semiquantitative analysis of fungal communities that is far more detailed and extensive than those provided by previous methods such as the culture-dependent approach.

3.1.3 Aims of this study

Following an initial survey of fungi associated with cabbages in New Zealand and how to isolate them (Chapter 2), this study aimed to investigate fungal consortia associated with cabbage tissues, from three regions in New Zealand.

It was expected that both isolation and HTS would target overlapping but different fungal communities, where HTS would detect non-culturable or difficult to grow fungal taxa, and conversely isolation through culture could recover some fungi that can go undetected by DNA sequence–based approach.

This study further investigated whether the endophytic fungal communities were different in various plant tissues or geographical regions.

3.2 Materials and methods

3.2.1 Isolation of putative endophytes in 2016

Cabbage plants with no evidence of the presence of a pathogen from three different regions in New Zealand were collected in January 2016 (Figure 3.1). In total 24 cabbage plants were sampled from four fields in each region (two cabbage plants from each field). The isolation of putative endophytic fungi was done by fragment plating. Tissue fragment preparation followed the methods described in Chapter 2.

Figure 3.1: Map showing the three regions in New Zealand where cabbage plants were sampled (Canterbury – circle, Nelson – triangle and Pukekohe – square).

Multiple media plating

Four different types of media were used for multiple media plating, namely PDA, quarter strength PDA, SDA and AMM [54]. Key contents of the media in g/l are described in Table 3.1. One additional type of media was used in current study compared to the study described in Chapter (2) to extend the range of nutrient content in growing media and to possibly induce sporulation in recalcitrant isolates. Media as diluted as 1/10-strength PDA were used to induce sporulation of plant pathogens *in vitro* [126].

Chloramphenicol (100 mg/l) and Streptomycin (350 mg/l) and Tetracycline (50 mg/l) were added to all the media to suppress bacterial growth. Each medium was used with and without the addition of Rose bengal (100mg/l), thus gaining 8 different types of media. Twenty four segments were prepared for each type of plant tissue (roots, stems and leaves) and placed in multiwell plates, six per plate. In total 1728 fragments were plated in multiwell plates.

Incubation of the fungal isolates and morphotyping and DNA extractions followed the methods described in Chapter 2.

	Dextrose	Peptone	Potato starch
1/4 PDA	5	0	1
AMM	10	0	0
PDA	20	0	4
SDA	40	10	0

Table 3.1: Key contents of potato dextrose agar (PDA), quarter strength PDA, Sabouraud dextrose agar (SDA) and Aspergillus minimal medium (AMM) in g/l.

3.2.2 High-throughput sequencing

In addition to the cabbage plants collected for isolation of fungal endophytes described in Section 3.2.1, another 24 cabbage plants adjacent to those collected were taken as a duplicate, thus totaling 48 plants for high-throughput sequencing detection of putative endophytic fungi (Figure 3.1).

HTS sample preparation

Fragment preparation followed the methods described in Chapter 2. Fragments were stored in DMSO at $-80°C$ until DNA was extracted several months later.

DNA extraction

DNA extraction followed a slightly modified protocol described in Allen et al. [3]. Briefly, frozen plant tissue was ground in a pre-cooled mortar with addition of liquid nitrogen to facilitate the grinding. Pre-heated cetyltrimethylammonium bromide based extraction buffer was added and the samples were incubated for 30 minutes at 65°C. After centrifuging, the supernatant was mixed with phenol:chloroform:isoamyl alcohol solution and centrifuged to precipitate proteins. The aqueous phase of the mixture was mixed with cold isopropanol and centrifuged to precipitate DNA. The pellets were resuspended in trisaminomethane-ethylenediaminetetraacetate based buffer with RNase and incubated at 37°C for 30 minutes and afterwards, cold ethanol was added and samples centrifuged. The resulting DNA pellets were washed with cold 70% ethanol before air drying them and resuspending in PCR-grade H_2O.

PCR

To amplify the ITS2 region of fungi while discriminating against cabbage ITS DNA, two-step nested PCR was performed with the first reaction amplifying the whole ITS region and the second targeting the ITS2 region.

First PCR reaction used

forward primer ITS1F (3' CTTGGTCATTTAGAGGAAGTAA 5') [39] and

reverse primer ITS4-fun (3' AGCCTCCGCTTATTGATATGCTTAAR 5') [147].

The following reaction used primers with adapter sequences allowing enzymatic label sequence added for HTS. In this reaction modified forward 58S-fun primer

(3' AACTTTYRRCAAYGGATCWCT 5') [132] and ITS4-fun reverse primer were used.

DNA purification and quality check

DNA amplicons were purified using an Agencourt© AMPure XP PCR purification kit following the manufacturer's instructions [15]. Briefly, a solution of magnetic particles was used to bind fragments of DNA longer than 100 base pairs (bp) and the short, low quality DNA fragments are discarded in a series of reactions. DNA content of amplicons was measured on a Qubit 2.0 Fluorometer™ (Life technologies) according to the manufacturer's instructions and sent for sequencing on Illumina MiSeq™ 2x250 PE to Massey Genome Service, Massey University, New Zealand.

Raw sequence data to community matrix

Demultiplexed forward and reverse sequence reads were merged using the "USEARCH" merging algorithm with default settings [31]. Primer and adapter sequences were removed from merged sequences using the "cutadapt" tool [78] and sequences with low quality were subsequently filtered out using the "Vsearch"" tool with more than one expected error and a minimal sequence length of 200 [108]. Sequences were clustered to OTUs based on 97% similarity using the "UPARSE" tool with default settings. Clustering algorithm also detects and labels chimeric sequences that are a likely product of sequencing errors [32].

The OTUs were assigned the most closely-related fungal species in the UNITE (accessed on 27.12.2016) and NCBI RefSeq Targeted Loci Project (accession number PRJNA177353, accessed on 27.12.2016) databases using the BLAST [4] program. Priority was given to matches from the UNITE database unless the best match was an unidentified taxon, in which case a match from the NCBI database was used.

Statistical analyses of fungal communities

Predicted species richness was calculated by Chao2 estimator [24]. A species-accumulation curve as well as predicted species richness were based on species abundance for both isolation and HTS approaches and were calculated using the function "specpool" in "vegan" package [87]. Richness of fungal communities was described by α and β diversity indices, where α diversity represented the average number of fungal species per plant to compare differences in regions, or average number per each type of host plant tissue (leaves, stem and roots) to compare those. The β diversity index compared homogeneity of group dispersions and was subsequently tested for differences by the Tukey HSD test using the "betadisper" function in the "vegan" package [87].

The distribution of shared or unique fungal taxa between types of plant tissue or regions sampled was displayed in Venn diagrams. To test whether such distribution was expected by chance, a null expectation model was based on the obtained data using the "permatfull" function in "vegan" package [87]. Isolate numbers for each species and each tissue type were kept constant in the simulated community matrices

for isolation dataset. Number of observed species was conserved across samples, as well as the number of samples where each species was observed in the HTS dataset.

Differences between fungal community composition based on detection approaches were tested using a permutational MANOVA test with the "adonis" function in "vegan" package [87]. All statistical calculations were performed in R [99]. A working example of R code is provided in a separate file deposited at Lincoln University Data Centre (Appendix B - R scripts. doi: 10.25400/lincolnuninz.9122549).

3.3 Results

3.3.1 Detected fungal taxa and their distribution

From metabarcoding 96 samples (from 48 cabbage plants divided above and below-ground), 395 OTUs were detected which were subsequently assigned to 314 species in 213 genera.

From 24 cabbages cut into 1728 fragments, 677 isolates representing 96 species in 55 genera were recovered. All isolates recovered along with additional information are summarised in a separate file deposited at Lincoln University Data Centre (Appendix D - Endophytic fungi from cabbage in New Zealand 2016. doi: 10.25400/lincolnuninz.9122504).

Species detected by both methods

Forty two species were detected by both HTS and isolation from tissue fragments. These species were the most abundant in both datasets as they represented 61% of all sequences obtained by HTS and 80% of all isolations.

The composition of both datasets was dominated by a few species including the ones detected by both methods, as can be seen on ranked abundance plots in Figures 3.2 – 3.4 for HTS and Figure 3.5 for the isolation dataset.

The relatively high number of species detected by only one of the two methods could be attributed partly to taxon assignment to a sequence from either dataset – since the HTS dataset was based on the ITS2 region while the isolation dataset was based on the whole ITS region. This resulted in different taxa being assigned to even highly similar sequences.

Thirteen OTUs from HTS dataset assigned to 12 species names that were not present in the isolation dataset had $>$ 200bp alignment and $>$ 97% similarity with a sequence obtained from a cultured fungus (Figure 3.6a).

Conversely 64 cultured isolates (represented by 50 unique sequences) were assigned to 22 species names that were not found in the HTS dataset, even though sequences of these isolates had $>$ 200bp alignment and $>$ 97% similarity with a sequence representing an OTU in the HTS dataset (Figure 3.6b).

Overall taxonomic composition

At the phylum level the taxonomic composition in the HTS and culture datasets was roughly similar with the dominant phyla Ascomycota and Basidiomycota rep-

resenting 96.9% and 2.6% of the OTUs from HTS and 93.1% and 6.1% of isolated fungal strains (Figure 3.7). However, fungi from the Zygomycota phylum were isolated relatively more frequently than they were detected by HTS (0.9% of isolations and 0.004 % of OTUs found only in one sample) and no *Mucor* species were detected with HTS, whereas four strains were isolated from 3 plants. On the other hand, fungi from the phylum Chytridiomycota were detected in almost half of the samples from HTS and represented 0.46 % of all OTUs, whereas there were no isolates belonging to this phylum.

Species accumulation

Species accumulation curves and Chao2 estimates of total species richness suggest undersampling for both methods (Figure 3.8). In total 473 species were expected to be found according to data from HTS, whereas the culturing approach suggests the species richness could be 162.

3.3.2 Communities in respect to site and plant part

Differences in the composition of fungal communities could be explained mainly by the origin from above or below ground tissues for the HTS dataset, while the isolation dataset added more resolution separating above-ground communities clearly between stem and leaf associated fungi. Furthermore the composition of fungal communities was different in three sampling regions in New Zealand according to both datasets.

Whether a fungal community detected by HTS was from the above or below ground part of the cabbage plant explained 17.6% of the differences between communities, while the region where the host cabbage was grown explained 7.5% and the interaction between these two factors explained an additional 4.4% of the variability at $p = 0.001$ (Figure 3.9).

Similarly fungal communities detected by isolation through culture were different in the various tissues from which they were isolated as well as region where the cabbage plant was grown (tissue effect was 24.8% at $p = 0.001$ and the region and the interaction both were 7.3% at $p < 0.01$). However, some of the cabbage tissue samples had no fungi detected (2 stems and 1 root sample from Nelson and 1 root sample from Pukekohe) (Figure 3.9).

Communities in respect to site

Fourteen species were isolated from all three regions (Figure 3.10). Although more species were expected to be shared between the regions, those shared were some of the most abundant and included *Alternaria alternata*, *Boeremia exigua*, *Cladosporium exasperatum*, *Fusarium circinatum*, *Fusarium equiseti*, *Fusarium sporotrichoides*, *Monographella cucumerina*, *Plectosphaerella oratosquillae*, *Pyrenochaeta lycopersici* and *Stemphylium herbarum*. Although isolated from all three regions, most of the *Alternaria alternata* strains originated from Nelson, while *Fusarium equiseti* was recovered from Nelson more than from Pukekohe. Distribution of species in the sampled regions is summarised on a heat map in Figure 3.11.

Similarly, 88 out of 314 species were detected in all three sampled regions by the sequence based approach (Figure 3.12). These were again some of the most abundant species across the whole dataset and include *Acremonium alternatum*, *Alternaria eichhorniae*, *Boeremia strasseri*, *Cladosporium exasperatum*, *Chalastospora ellipsoidea*, *Didymosphaeria variabile*, *Fusarium circinatum*, *Fusarium equiseti*, *Juncaceicola alpina*, *Monographella cucumerina*, *Plectosphaerella alismatis Pyrenochaeta lycopersici*, *Stemphylium herbarum* and *Tetracladium setigerum*. Again some of these species were unevenly distributed, as was the case with *Didymosphaeria variabile* and*Monographella cucumerina* detected mainly in samples from Pukekohe, *Alternaria eichhorniae* in Nelson or *Chalastospora ellipsoidea Ilyonectria sp.* in Canterbury (Figures 3.13 – 3.16).

The trends in α diversity were not consistent between HTS and isolation, as the lowest average number of species per plant was detected in Pukekohe by HTS ($p < 0.01$) but this was not the same for the isolation dataset. However β diversity indices showed similar patterns for both methods where plants from Nelson had a significantly different β diversity than those in Canterbury ($p < 0.04$). (Table 3.2).

	Species (total)		α diversity		β diversity	
Region	HTS	Isolation	HTS	Isolation	HTS	Isolation
Pukekohe	163	51	37.06*	12.75	0.3626	0.447
Nelson	202	53	47.88	13.63	0.3414	0.376
Canterbury	204	40	44.88	10	0.3809	0.4943

Table 3.2: Diversity indices for sampled regions and detection methods.

Communities in respect to plant part

The highest average number of species per plant was isolated from stems ($p = 0.043$), followed by leaves and roots. In contrast there was no significant difference between the number of species detected by HTS above and below ground. There were no differences in β diversity of plant tissues as detected by both methods (Table 3.3).

	Species (total)		α diversity		β diversity	
Plant part	HTS	Isolation	HTS	Isolation	HTS	Isolation
Leaves/Stems	235	46/54	26.73	4.41/6.33*	0.425	0.5209/0.506
Roots	230	41	26.44	4.13	0.4335	0.5172

Table 3.3: Diversity indices for sampled plant parts and detection methods.

Nine species were isolated from all three plant parts, which was lower than expected by chance (Figure 3.17). Fungi, such as *Monographella cucumerina* and *Plectosphaerella oratosquillae*, were recovered evenly from various plant tissues. Other taxa, although isolated from all three tissues, were predominnantly recovered from a certain tissue. *Stemphylium herbarum* and *Cladosporium exasperatum* were isolated mostly from leaves and stems and *Boeremia exigua* was isolated virtually

entirely from stems, except there was one isolate of each species originating from roots. *Pyrenochaeta lycopersici* and *Fusarium circinatum* were isolated predominantly from roots (Figure 3.18).

The sequence-based approach detected 151 species shared in above and below ground samples, which was slighlty less than expected by chance (Figure 3.19). Consistent with the dataset obtained from isolation, some of these fungi were evenly distributed (*Monographella cucumerina*, *Plectosphaerella alismatis*) or predominantly detected in samples from above ground tissues (*Acremonium alternatum*, *Alternaria eichhorniae*, *Ascochyta phacae*, *Chalastospora ellipsoidea*, *Cladosporium exasperatum*, *Entyloma cosmi*, *Erysiphe hypophylla*, *Gibellulopsis piscis*, *Stemphylium herbarum* and *Ulocladium chartarum*) or below ground tissues (*Fusarium circinatum*, *Fusarium oxysporum*, *Ilyonectria sp. Juncaceicola alpina* and *Pyrenochaeta lycopersici*) (Figure 3.20 – 3.23).

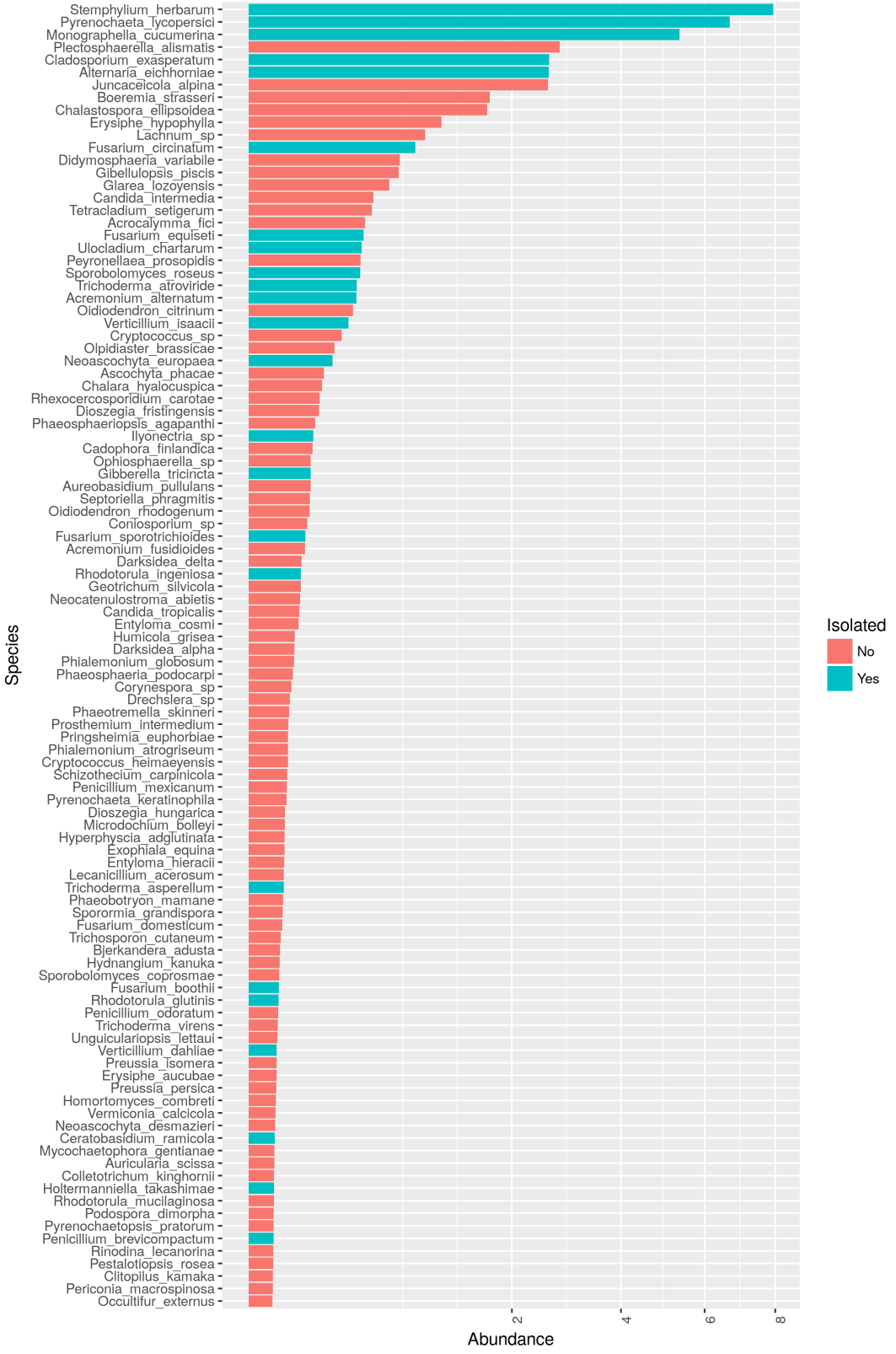


Figure 3.2: Species ranked by the abundance (sum of frequencies) in samples (squareroot scale) as detected by high-throughput sequencing (HTS). Species detected only by HTS are represented by red bars while species also detected by isolation (Figure 3.5) are in turquoise (Continued on next page).

Figure 3.3: (Continued from previous page) Species ranked by the abundance (sum of frequencies) in samples (squareroot-scale) as detected by high-throughput sequencing (HTS). Species detected only by HTS are represented by red bars while species that were also isolated (Figure 3.5) are in turquoise (Continued on next page).

Figure 3.4: (Continued from previous page) Species ranked by the abundance (sum of frequencies) in samples (squareroot-scale) as detected by high-throughput sequencing (HTS). Species detected only by HTS are represented by red bars while species that were also isolated (Figure 3.5) are in turquoise.

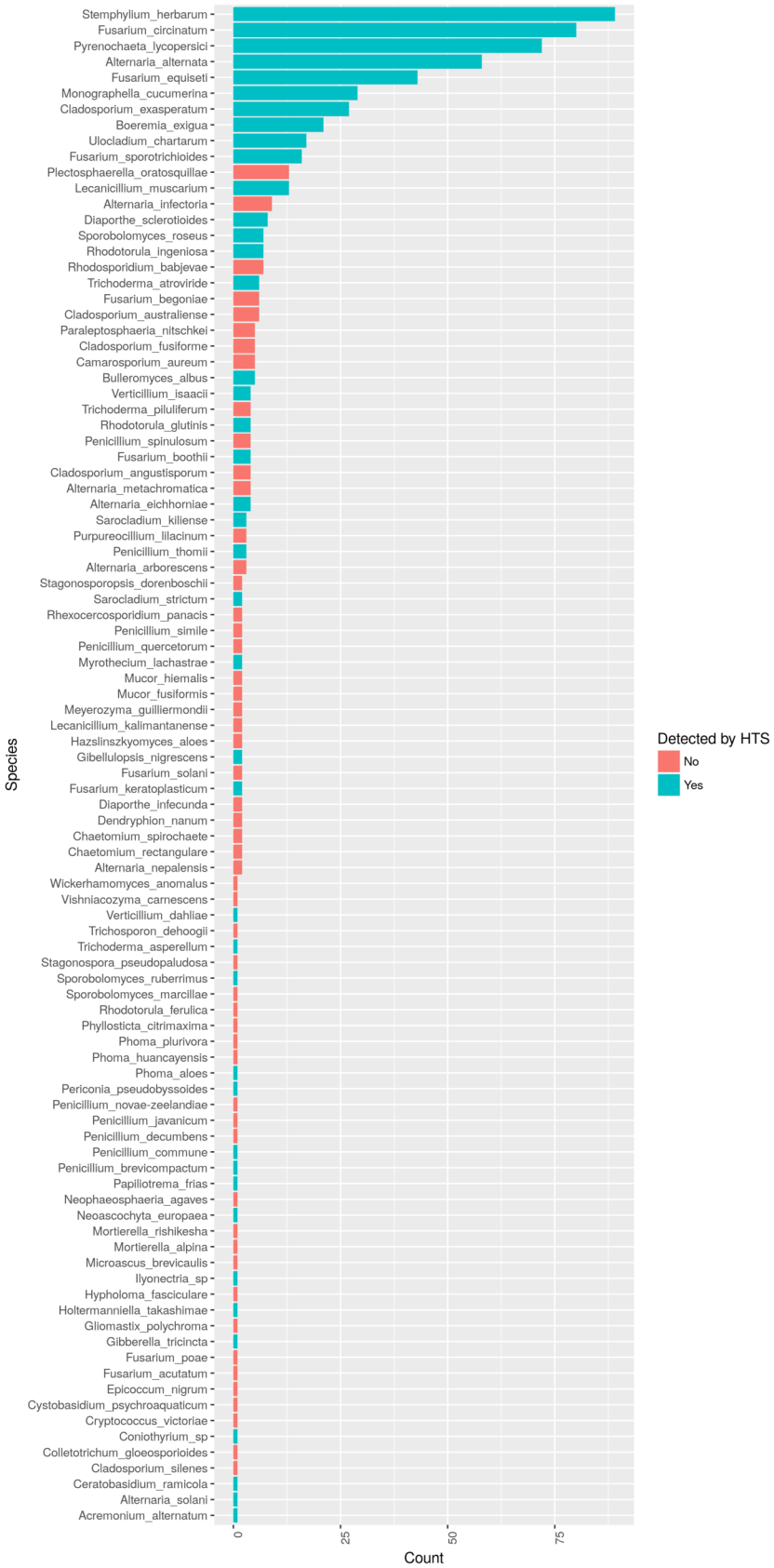
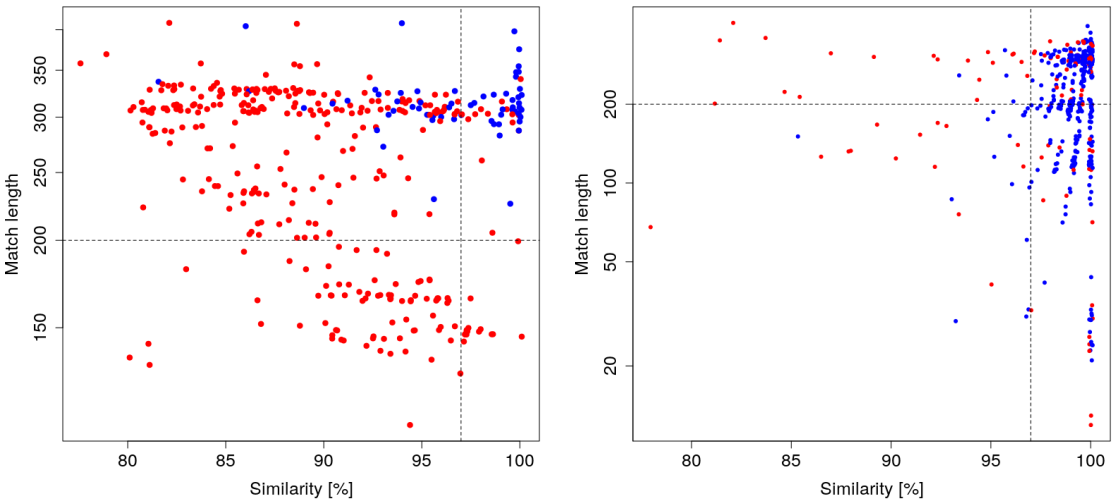


Figure 3.5: Species ranked by the total number of isolates. Those detected only by isolation are represented by red bars while species also detected by HTS (Figures 3.2 – 3.4) are turquoise.



(a) HTS matched to isolation

(b) isolation matched to HTS

Figure 3.6: Length and similarity of a BLAST match of sequences from either HTS or isolation dataset with the closest match from the other dataset. Sequences that were assigned species names found in both datasets are blue, while red circles represent sequences that were assigned taxons unique for respective method of detection.

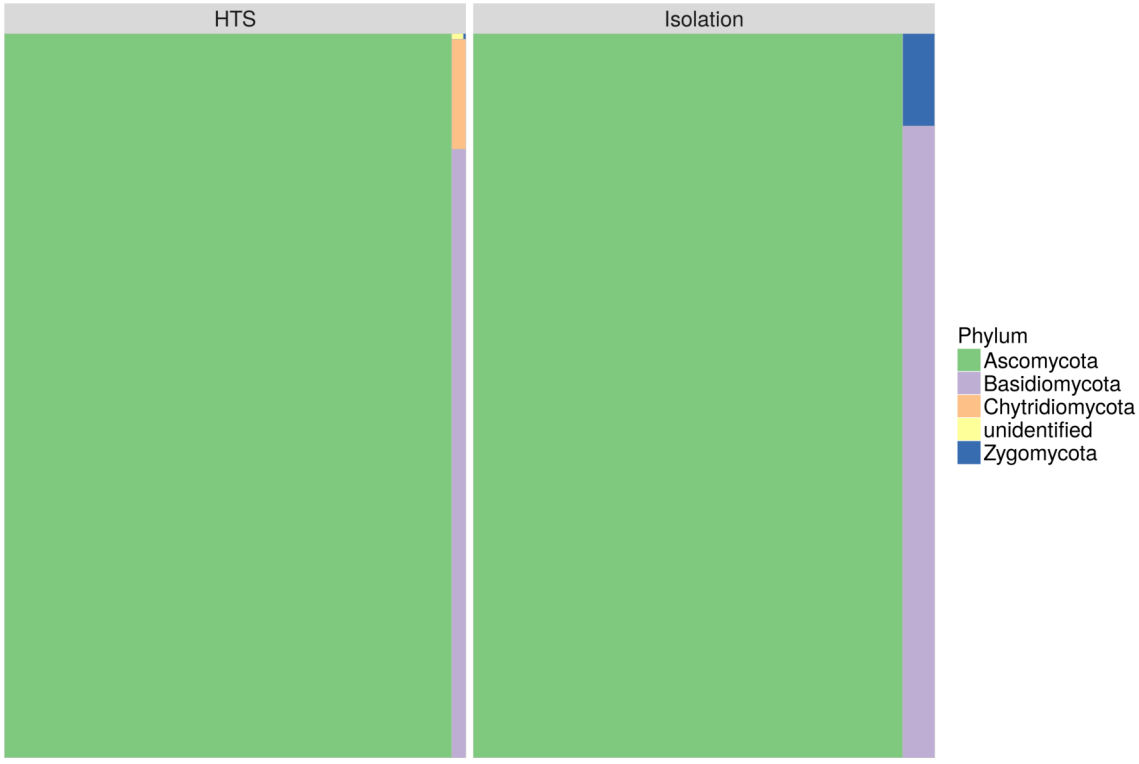


Figure 3.7: Phylum composition (rectangle area represents fraction of sequences from HTS or strains from isolation) of datasets as obtained by HTS (left) and isolation (right).

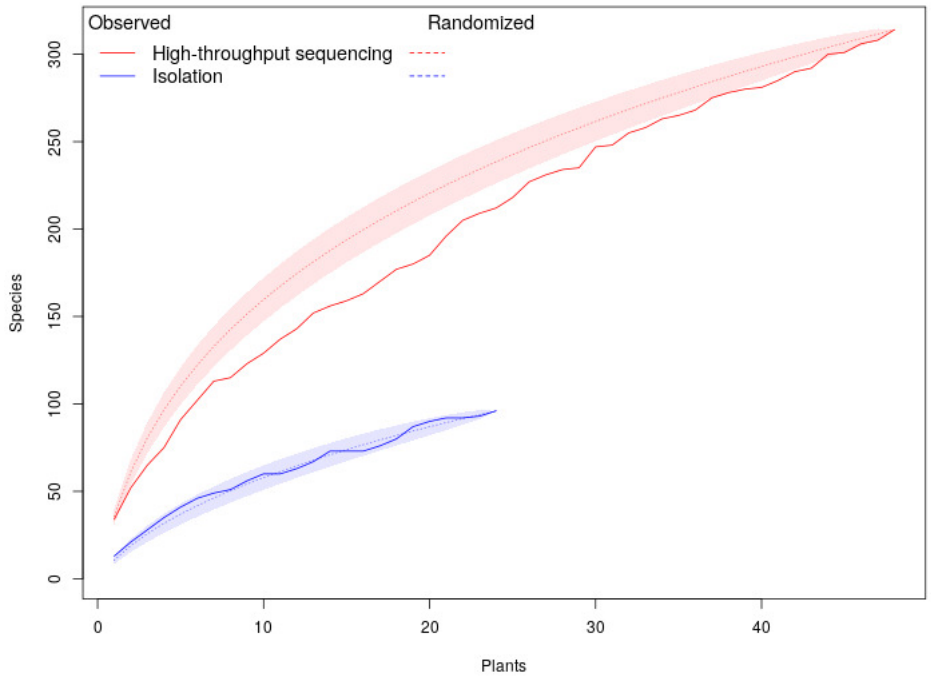
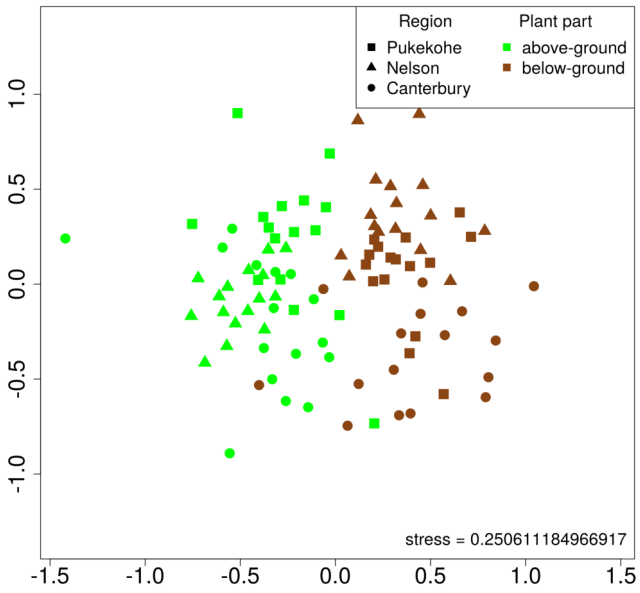
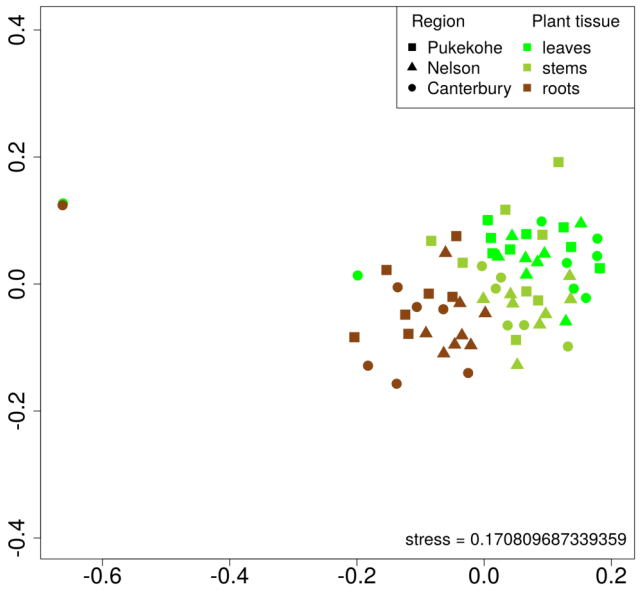


Figure 3.8: Species-accumulation curves plotted as obtained (solid line) and based on randomisation of samples (dashed line) with standard deviation (shaded area) are shown for high-throughput sequencing (blue) and isolation through culturing (red).

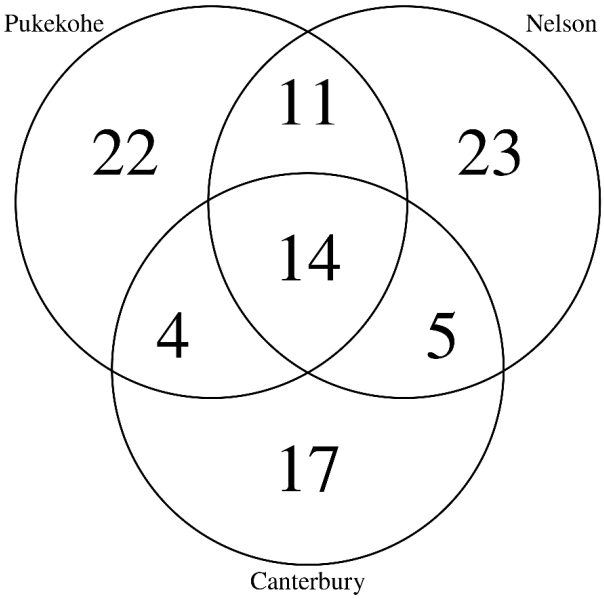


(a) Multidimensional scaling of fungal communities as detected by HTS

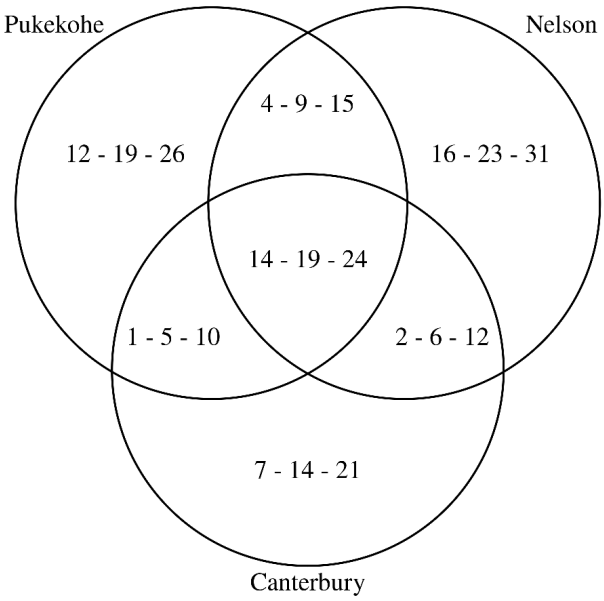


(b) Multidimensional scaling of fungal communities as detected by isolation

Figure 3.9: Multidimensional scaling of fungal communities as recovered from leaves (green), stems (olivegreen) or above ground tissues for HTS (green) and roots (brown) from 3 regions in New Zealand (Canterbury – circle, Nelson – triangle and Pukekohe – square).



(a) Observed



(b) Expected

Figure 3.10: Venn diagram showing observed(a) and expected(b) distribution of species in regions as detected by isolation. Expected numbers were based on random sampling of 9999 iterations with 95% confidence intervals shown (lower confidence interval - mean - upper confidence interval).

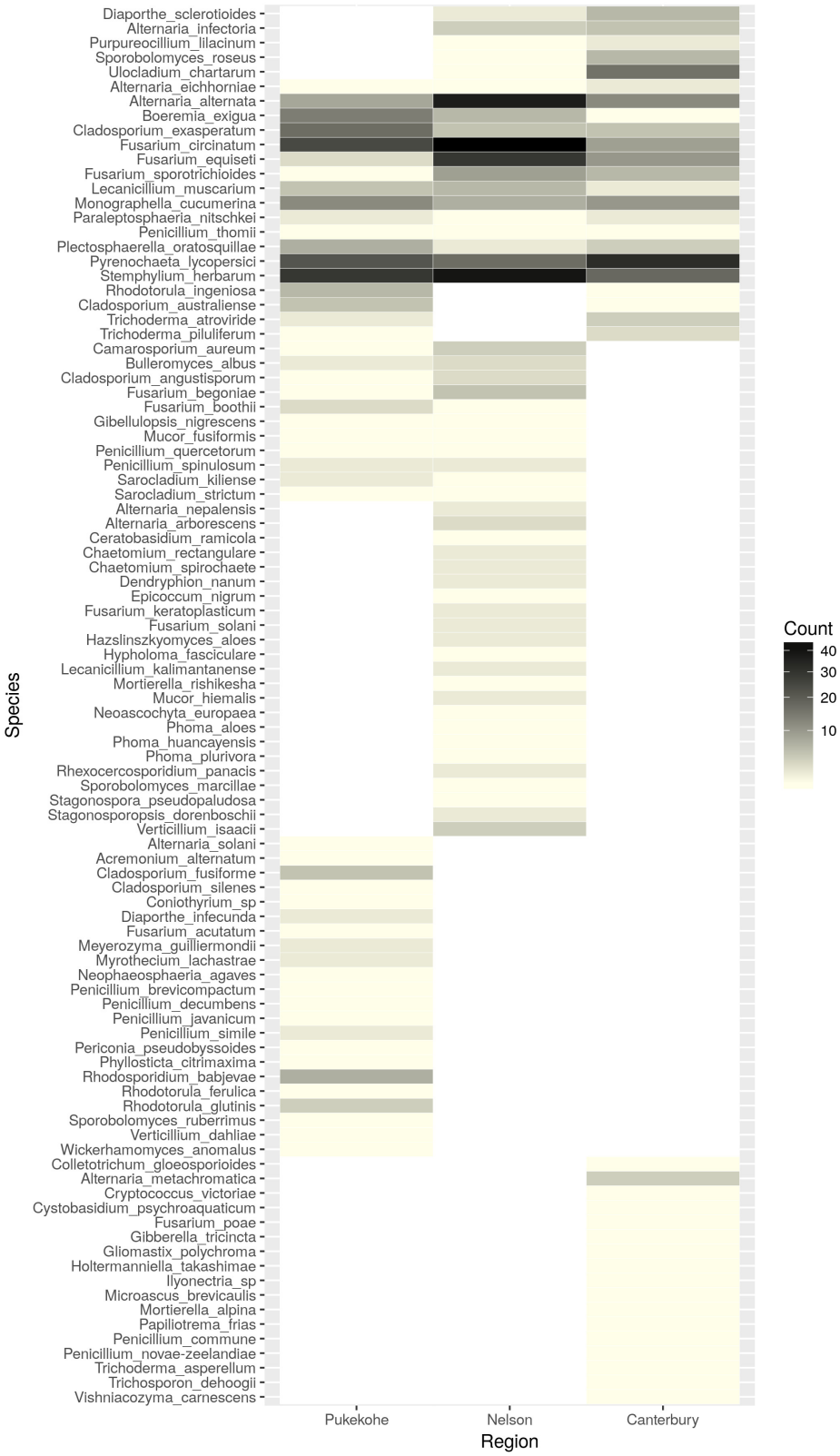
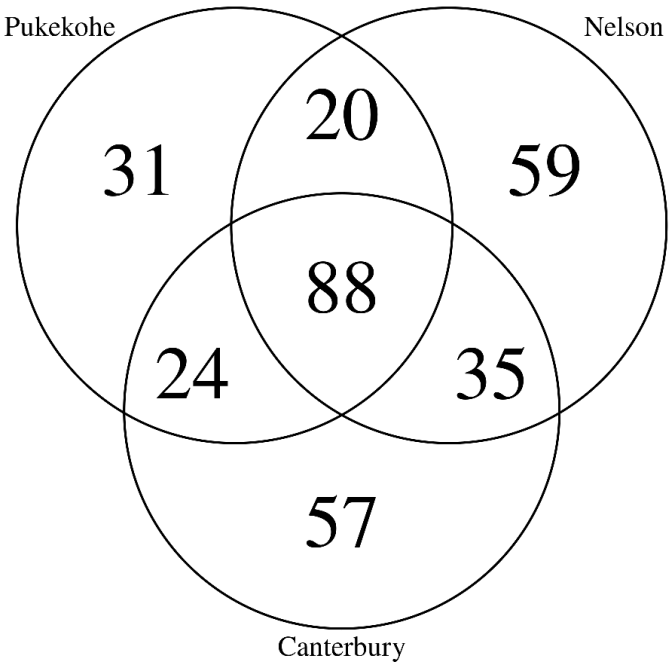
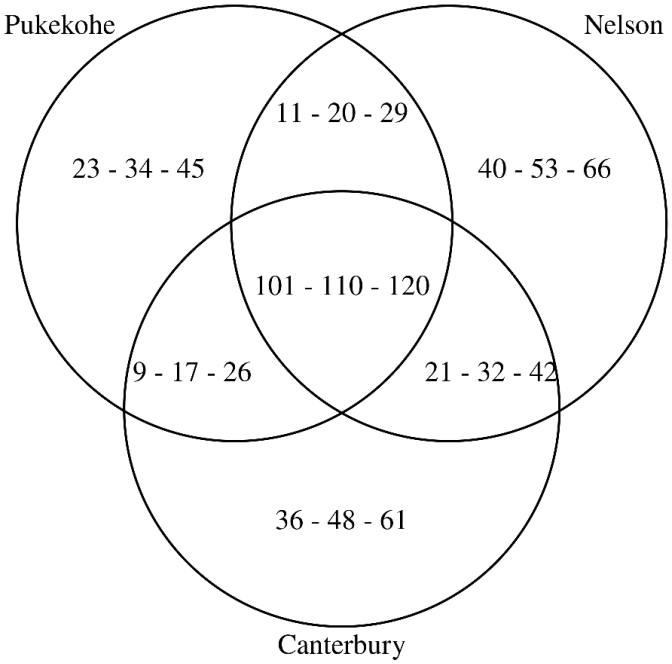


Figure 3.11: Heat map showing distribution of species in regions as detected by isolation.



(a) Observed



(b) Expected

Figure 3.12: Venn diagram showing observed(a) and expected(b) distribution of species in sampled regions as detected by high-throughput sequencing. Expected numbers were based on random sampling of 9999 iterations with 95% confidence intervals shown (lower confidence interval - mean - upper confidence interval).

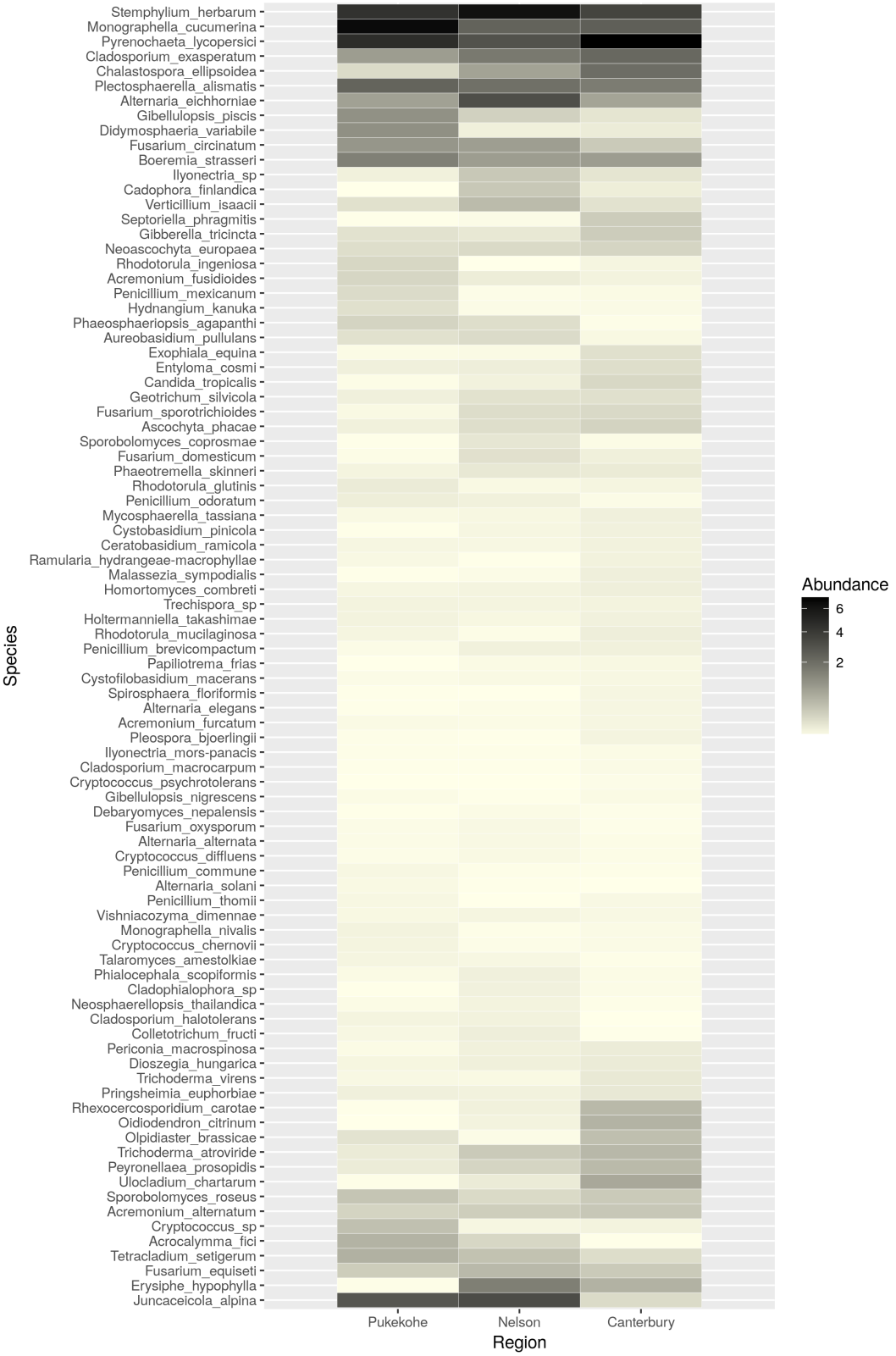


Figure 3.13: Heat map showing distribution of species in sampled regions as detected by high-throughput sequencing (Continued on next page).

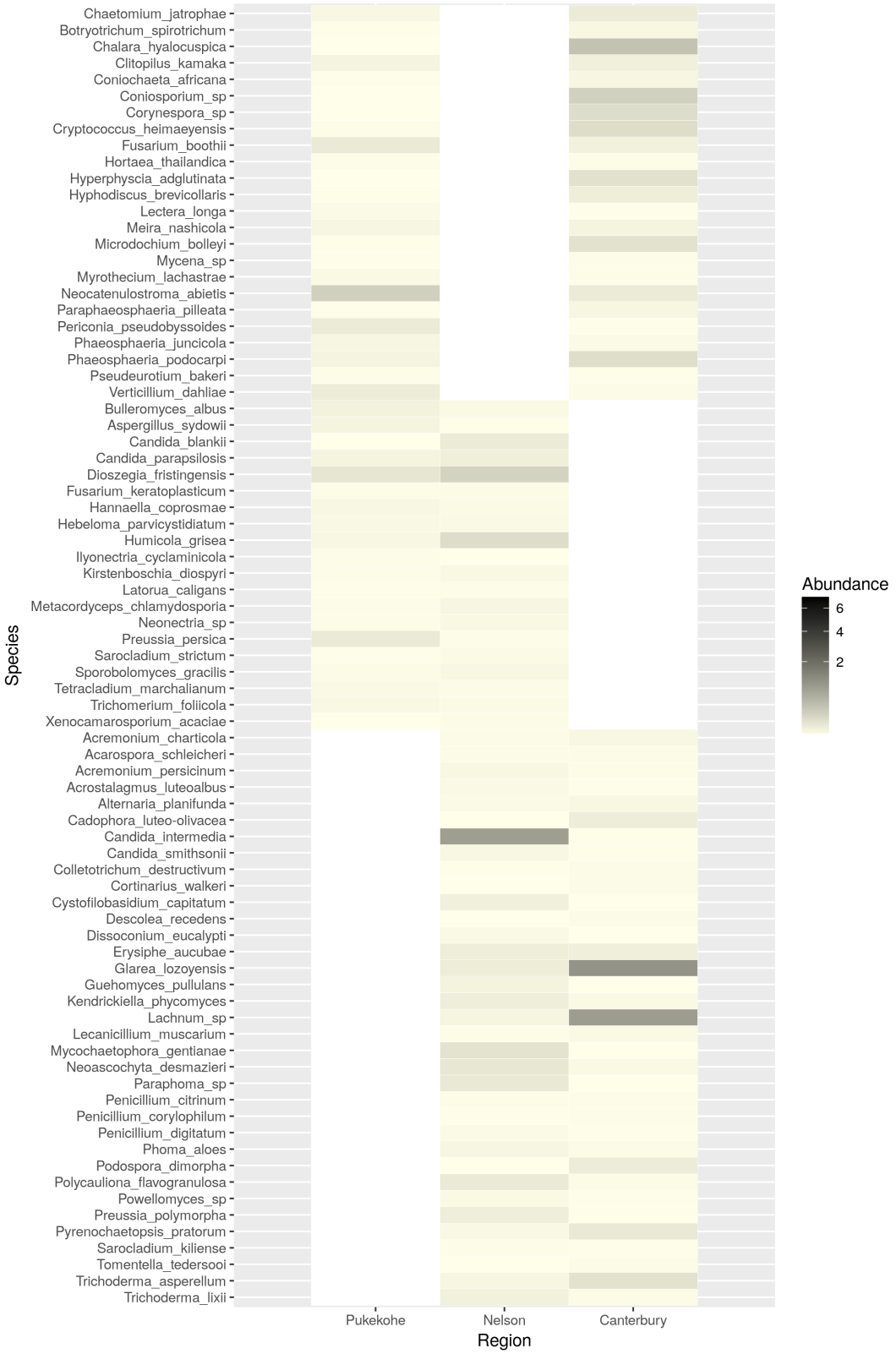


Figure 3.14: (Continued from previous page) Heat map showing distribution of species in sampled regions as detected by high-throughput sequencing (Continued on next page).

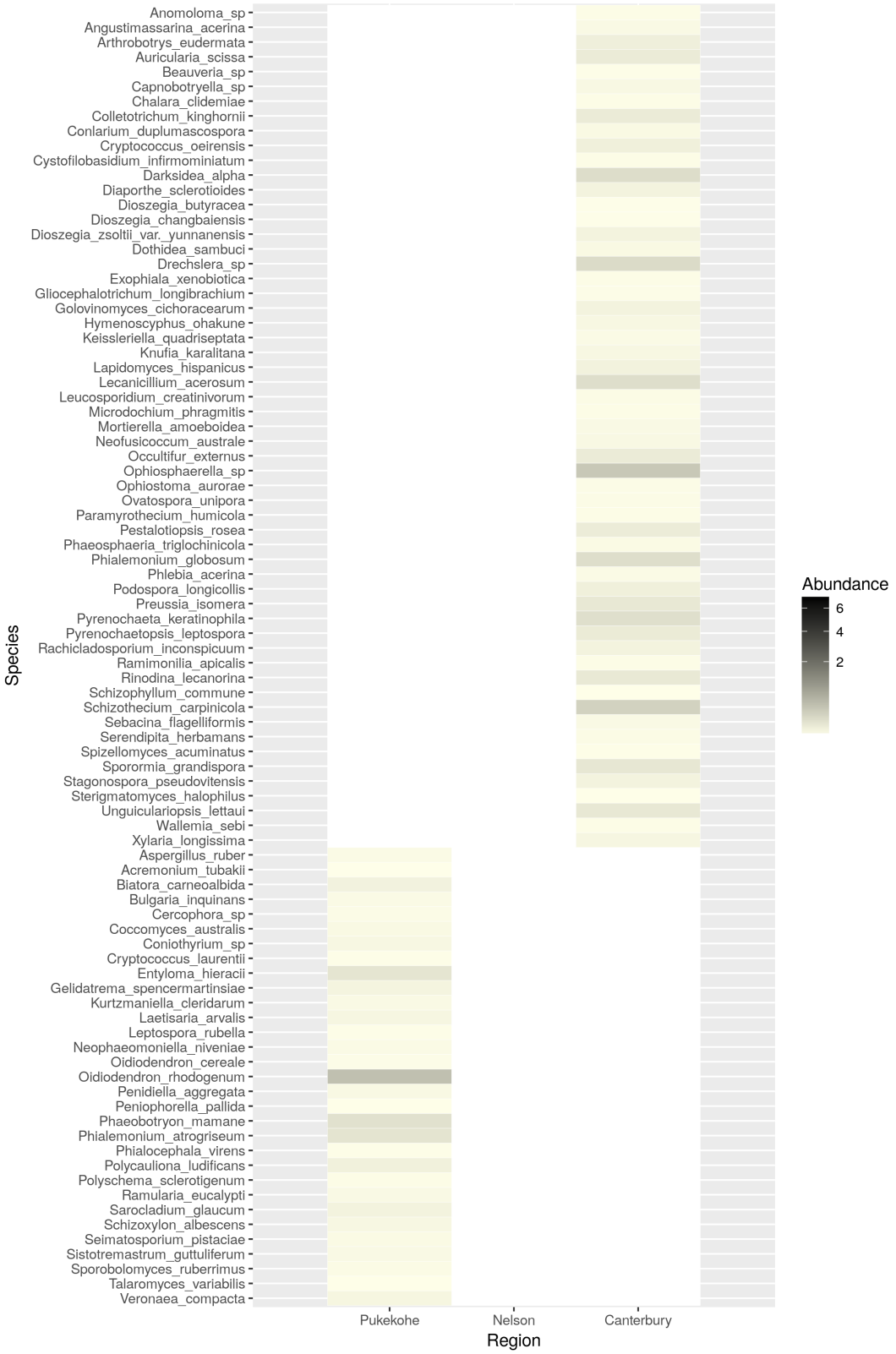


Figure 3.15: (Continued from previous page) Heat map showing distribution of species in sampled regions as detected by high-throughput sequencing (Continued on next page).

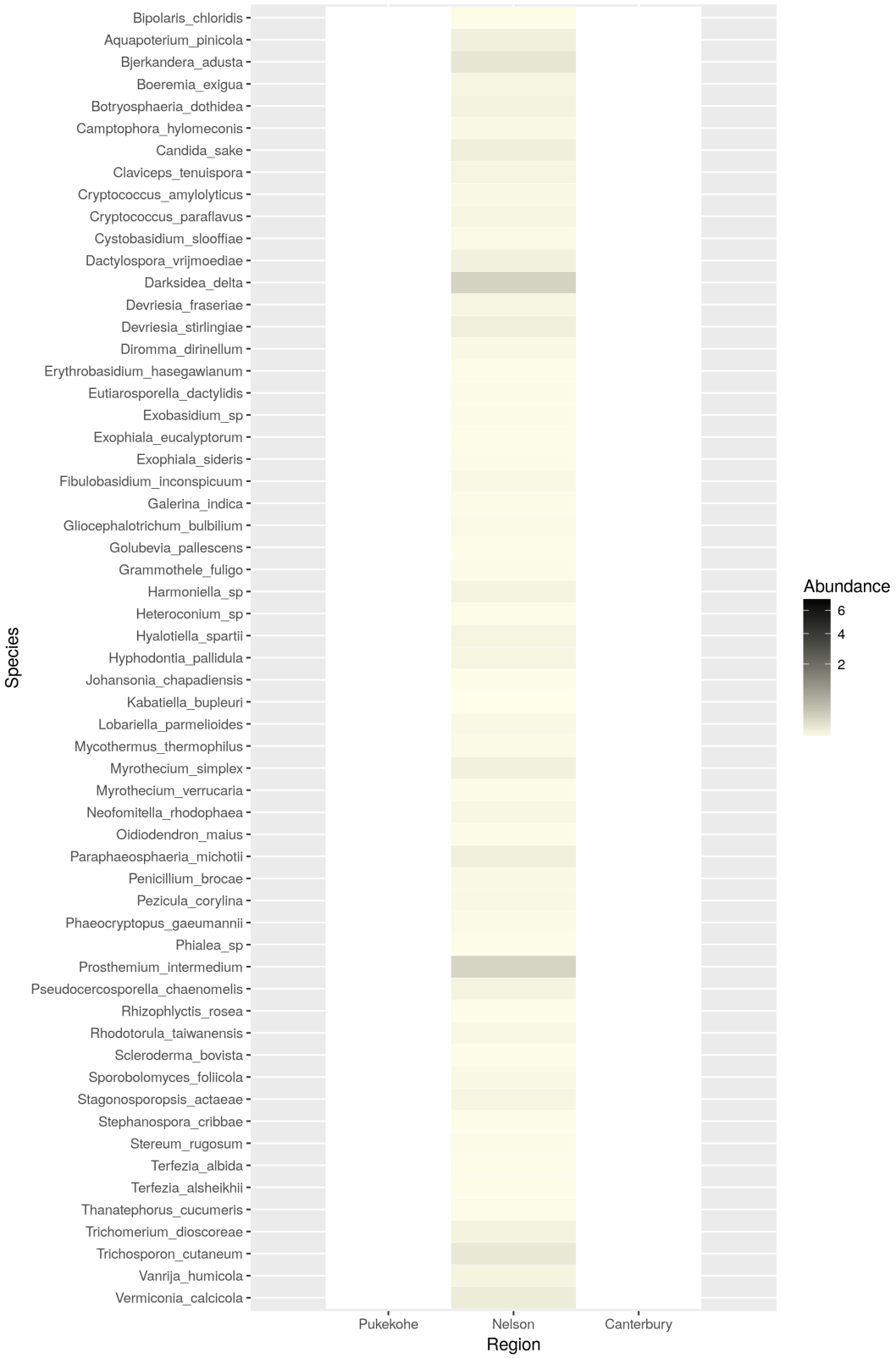
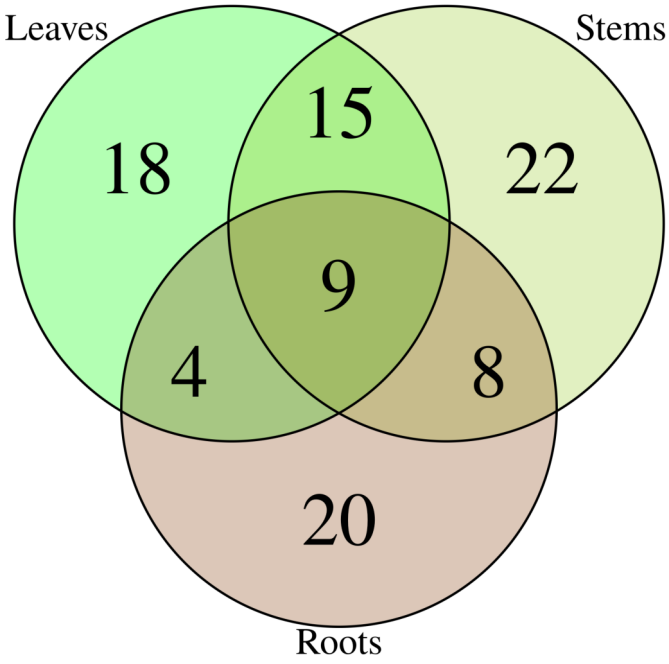
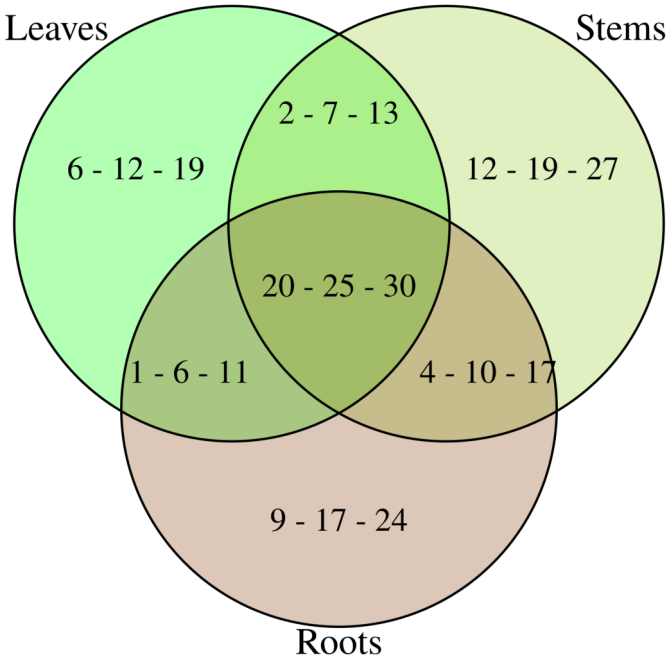


Figure 3.16: (Continued from previous page) Heat map showing distribution of species in sampled regions as detected by high-throughput sequencing.



(a) Observed



(b) Expected

Figure 3.17: Venn diagram showing observed(a) and expected(b) distribution of species in sampled plant parts as detected by isolation. Expected numbers were based on random sampling of 9999 iterations with 95% confidence intervals shown (lower confidence interval - mean - upper confidence interval).

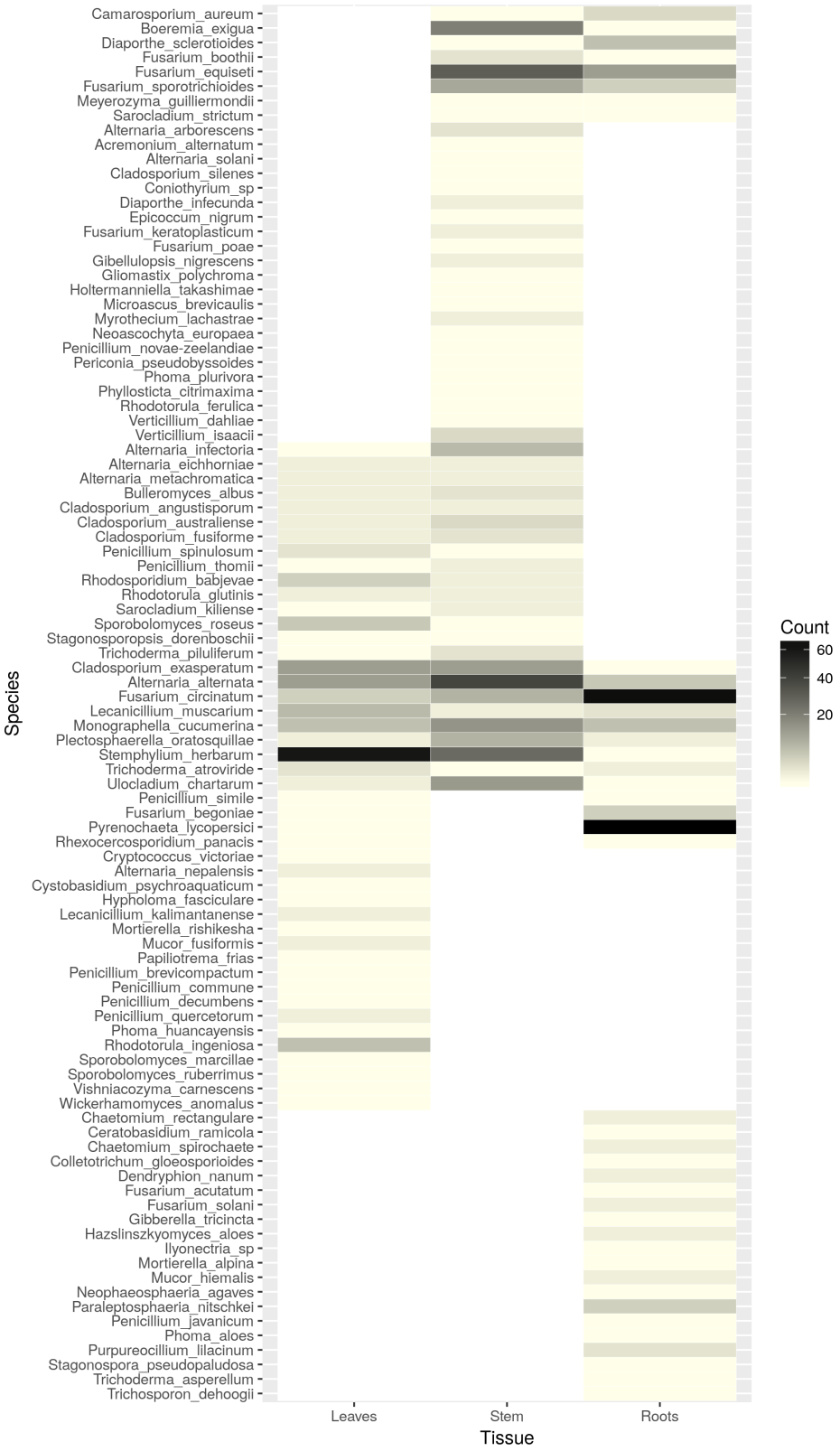
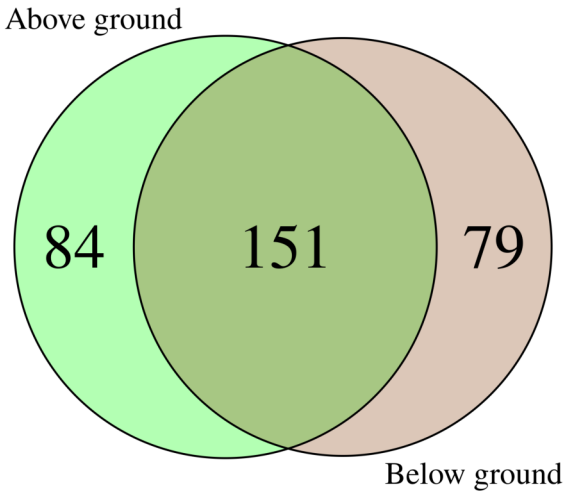
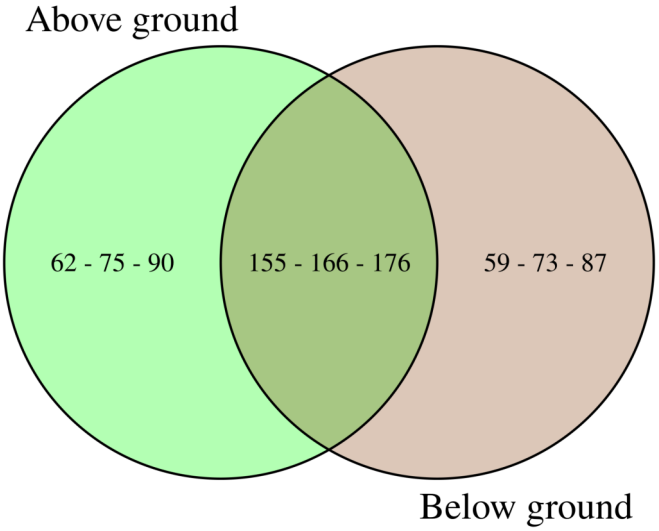


Figure 3.18: Heat map showing distribution of species in sampled plant parts as detected by isolation.



(a) Observed



(b) Expected

Figure 3.19: Venn diagram showing observed(a) and expected(b) distribution of species in sampled plant parts as detected by high-throughput sequencing. Expected numbers were based on random sampling of 9999 iterations with 95% confidence intervals shown (lower confidence interval - mean - upper confidence interval).

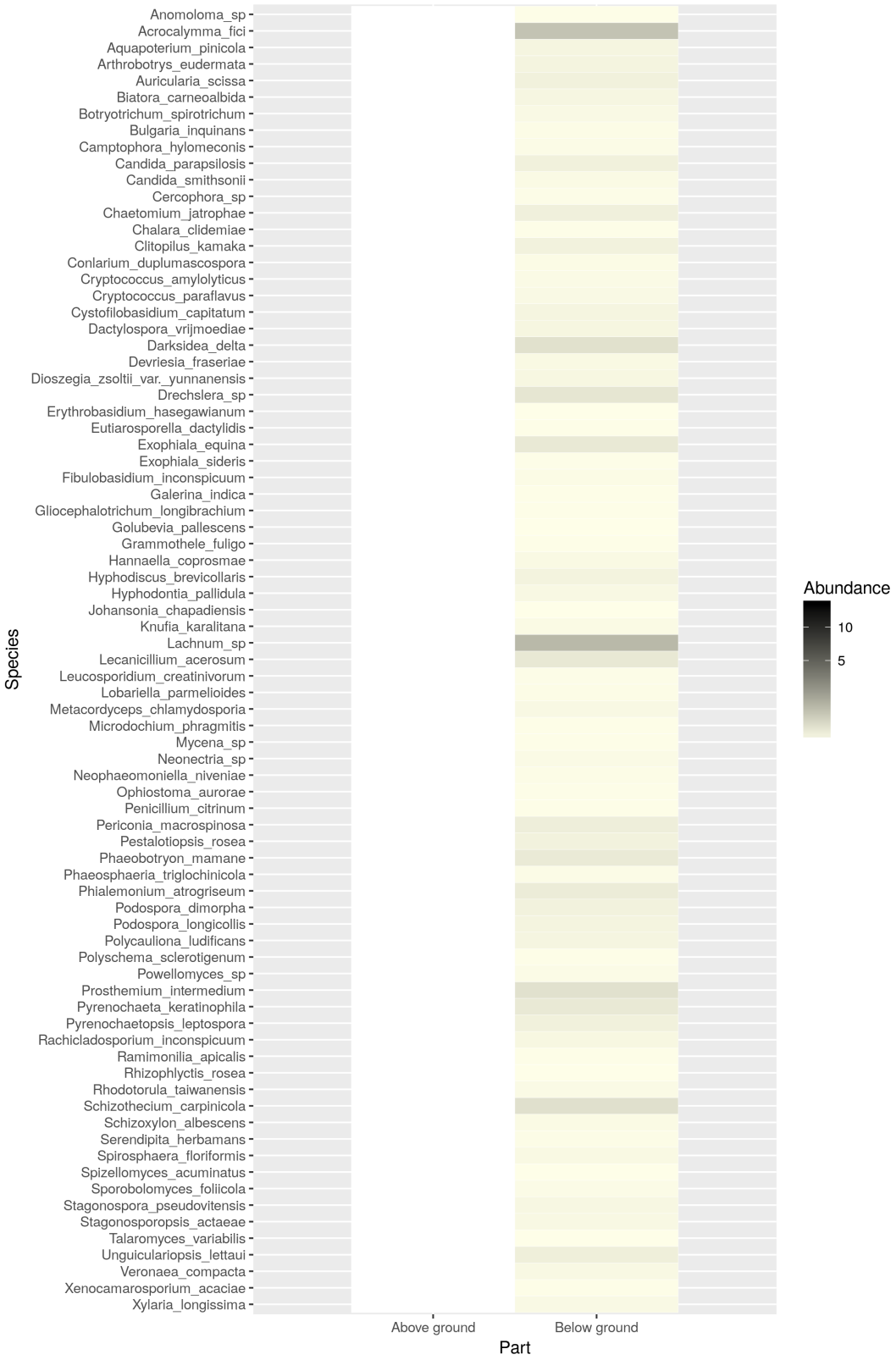


Figure 3.20: Heat map showing distribution of species in sampled plant parts as detected by high-throughput sequencing (Continued on next page).

Figure 3.21: (Continued from previous page) Heat map showing distribution of species in sampled plant parts as detected by high-throughput sequencing(Continued on next page).

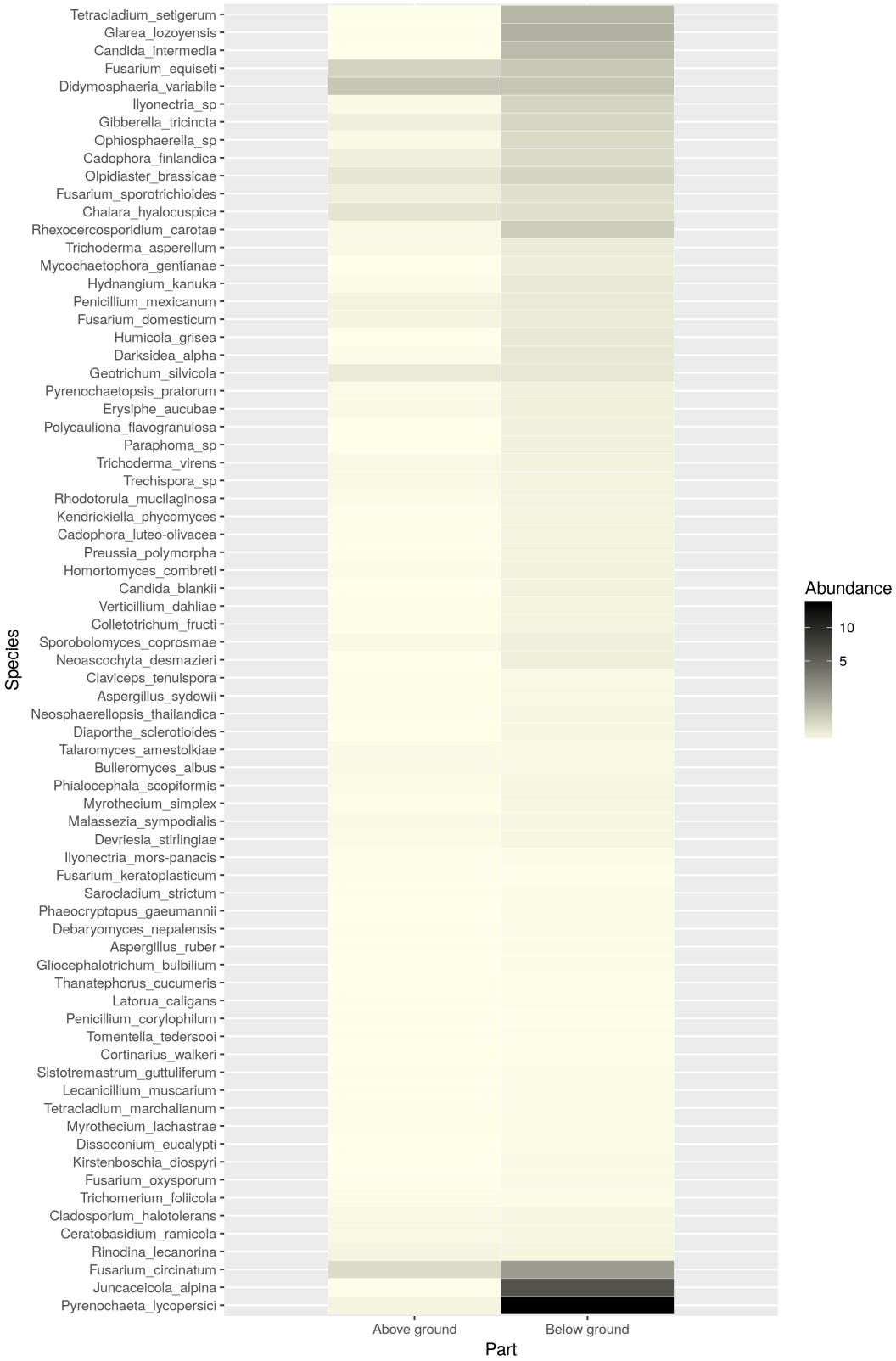


Figure 3.22: (Continued from previous page) Heat map showing distribution of species in sampled plant parts as detected by high-throughput sequencing (Continued on next page).

Figure 3.23: (Continued from previous page) Heat map showing distribution of species in sampled plant parts as detected by high-throughput sequencing.

3.4 Discussion

3.4.1 Fungal taxa detected

This study aimed to identify fungi associated with cabbage plants in three regions of New Zealand through sequence-based detection and isolation through culture. Ninety six species were isolated from 24 cabbage plants while 314 putative species (395 OTUs) were detected from 48 plants by sequencing.

What has been found in brassicas so far?

Many of the species detected in this study have been reported to form association with plants from the family Brassicaceae, to which cabbage belongs.

For example a recent study by Barret et al. [13] described sequence-based evidence of fungi such as *Alternaria*, *Cladosporium* or *Penicillium* to be associated with seeds and seedlings of several plants including Brassicaceae plants such as cabbage and oilseed rape. Interestingly, *Fusarium* was not a dominant genus in the endophytic communities associated with the Brassicaceae seeds or plants studied, contrary to the current study, where *Fusarium circinatum* and *Fusarium equiseti* were among the most abundant species either isolated or detected by HTS. On the other hand, *Fusarium* and *Alternaria* were found to be dominant genera from the community isolated from the roots of Brassicaceae in a study by Ishimoto et al. [62]. Furthermore, 40 species, the most abundant of which were species from the genera *Alternaria*, *Fusarium* or *Chaetomium*, were isolated from oilseed rape in China [153]. These data are consistent with findings in this study where *Alternaria*, *Cladosporium*, *Chaetomium* or *Fusarium* were the most abundant genera detected by both methods.

Why choose species over OTUs?

Many HTS studies work with operational taxonomic units (OTUs). OTU is a concept that helps dealing with large HTS datasets which usually contain hundreds of thousands of sequences. The 97% similarity threshold used widely to cluster sequences into an OTU is assumed to approximate resolution of sequence data to species level [75]. However, this approach has its trade-offs when sequences representing several species can get clumped together or, conversely, a single species is represented by several OTUs [71, 86].

In this study after initial OTU clustering of the sequences obtained by HTS, the clusters represented by a single sequence were assigned to species based on closest match to reference databases. Thus, the initial dataset of 395 OTUs was reduced to 314 species, suggesting that at least in some cases the 97% threshold was oversplitting the acquired HTS sequences. In some cases a species hypothesis from a database was a best match for several OTUs, however, with similarity lower than 97%. In this scenario even quite dissimilar OTUs could be clustered under a single species.

Nonetheless, to be able to compare datasets from isolation and HTS species level resolution was chosen.

Identification at the species level revealed differences between the two datasets, which could have been caused by detecting different species, but more likely this was the result of using two DNA markers (whole ITS for isolation dataset and ITS2 for HTS dataset), where even very similar sequences from either dataset were assigned to different taxa (Figures 3.6a 3.6b). Sequences of species from HTS dataset such as *Boeremia strasseri*, *Cladosporium halotolerans*, *Plectosphaerella alismatis* and *Penicillium odoratum* have high similarity to those in the isolation dataset, however, they were assigned to different species but the same genus in respective datasets. Sequences from HTS dataset assigned to species such as *Juncaceicola alpina* or *Chalastospora ellipsoidea* matched most closely to sequences from isoation dataset which represented a different genus – *Camarosporium aureum* and *Alternaria infectoria* respectively.

The above-mentioned taxa were among the most abundant in their respective datasets, and as such introduce uncertainty when comparing communities based on a single DNA marker.

The issue of DNA marker choice has been debated thoroughly with more and more metabarcoding surveys, such as this one, in the last two decades. The ITS region gained popularity and has been regarded as a universal barcode for fungal phylogeny [75, 17]. However,the limitations of HTS technology in respect to the length of a chosen barcode favor the use of a subregion of ITS (either ITS1 or ITS2), especially on the Illumina platform where higher sequencing depth is traded for shorter sequence length [132]. When choosing between ITS1 and ITS2, the latter seems to be a more sensible choice because of its lower variability in length and better representation in databases and other studies [75, 136].

Phylum

On a phylum level this study follows a trend in numerous other surveys where endophytic fungal communities were dominated by ascomycous fungi [9, 13, 64, 153]. However detection of Chytridomycota by HTS with no isolates belonging to this phylum reveal the limitations of the culture-dependent approach, whereas isolation of several *Mucor* strains but failure to detect any by HTS suggests primer or PCR bias.

3.4.2 Composition of fungal communities

The fungal communities detected in this study showed high segregation in different plant tissues in all sampled cabbage plants. High dissimilarity between above and below ground communities can be further disseminated when above ground tissues are split into leaves and stems. Somewhat less distinct separation of the fungal communities in cabbage plants is a result of the region in New Zealand where the cabbage plants were sampled.

Plant part

Several species in this study were detected only in above or below ground tissues or were preferentially isolated from roots, stems or leaves.

Tissue preference, however, seems to be a trait not common to all fungi found within a host plant. This was true for some frequently isolated fungi such as *Monographella cucumerina* and *Plectosphaerella oratosquillae*. Furthermore, claiming that certain rare species display tissue preference based on a few or even a single isolation would be lacking any statistical evidence. This applies also to the HTS dataset which is at most semi quantitative.

Therefore tissue preference can be concluded only for a few species in this study including those from the genera *Stemphylium* and *Cladosporium* which were isolated mostly from leaves and stems and *Boeremia* isolated from stems or *Pyrenochaeta* and *Fusarium* which were isolated mostly from roots. Sequence based approach revealed preferential above ground tissue localisation of *Acremonium*, *Alternaria*, *Ascochyta*, *Chalastospora*, *Cladosporium*, *Entyloma*, *Erysiphe*, *Gibellulopsis*, *Stemphylium* and *Ulocladium* whereas *Fusarium*, *Ilyonectria*, *Juncaceicola* and *Pyrenochaeta* were detected predominantly below the ground.

These findings align well with findings in the study described in Chapter 2 and studies on plant endophytes where distinct fungal communities were described in leaves, stems or roots. Zhang et al. [153] recovered most of their *Alternaria* isolates from leaves, and most of their *Fusarium* isolates from roots. Stems of the oilseed rape plants harboured the highest number of isolates, similarly to this study. Ishimoto et al. [62] found *Fusarium* and *Alternaria* to be dominant genera in the root endophytic community, however they did not attempt to describe above ground endophytic fungi. Sun et al. [128] found the species richness to be higher and the composition of endophytic fungal communities to be different in twigs compared with leaves of three temperate trees studied. Arnold [8] suggested that fungi often found in photosynthetic tissues such as *Altenaria alternata*, *Cladosporium cladosporoides*, *Chaetomium* or *Epicoccum pupurascens* can cope with the particularly harsh environment caused by abiotic stresses such as high fluctuation of moisture and UV light.

Tissue specificity is an important trait of endophytic fungal communities. Results presented in this study improve our understanding of the interactions between fungi and their host plants. Such information can be used and compared to assess ecological characteristics of endophytic fungal species. Apart from giving insights into possible evolutionary relations between fungi and plants [8], this could aid in targeted approach in manipulating microbial communities, which in turn has potential to improve current horticultural practices by developing novel microbiome-driven strategies [6, 5].

Region

It would be premature to draw conclusions about the drivers underlying differences in distribution of fungi across fields in New Zealand as only three regions with four fields in each were sampled. Although data on soil composition and climate were available in this study, the models trying to explain the variability of the endophytic fungal communities in respect to many of the soil properties and climate data would be inconclusive.

Nevertheless, there is a clear distinction between fungal communities in the three

sampling regions in this study as was detected in both isolated fungi and the HTS dataset. Regional patterns in endophytic fungal communities have been previously described by Arnold [8], who found that communities associated with pine needles differed markedly in species richness and composition in three geographic localities which were also differing in annual rainfall and other factors. Cevallos et al. [23] found that endophytic communities associated with epiphytic orchids were different between sites in lower altitudes but not in higher elevations.

The mechanisms by which endophytic fungal communities are affected in different geographical regions are yet to be untangled, with more studies aimed specifically on this issue. The underlying factors causing differences in endophytic communities could be harnessed in the development of biological control agents suited for specific region or in general for sustainable agriculture driven by microbiome manipulation [6, 5].

Other factors shaping communities?

On top of the unresolved mechanisms underlying regional differences in endophytic communities in cabbage, there may be other factors shaping the communities in New Zealand, that were not addressed in this survey.

One of the factors that could have driven the differences between sampled regions is field management, in a similar way as was described in a study with grass root endophytes, where composition of their communities changed due to agricultural management of temperate grasslands [148]. Interestingly, a study by Seghers et al. [114] found differences only in bacterial but not root fungal endophytic communities in maize as a result of different agricultural practices.

However, sampled fields in this study were under conventional agricultural management, where tilling, weeding, fertilizer and fungicide application were carried out in a similar manner across all regions.

Another factor that was not investigated in this study is the changes of fungal communities in various developmental stages of the host cabbage plants. Colonisation of host tissues is a dynamic process, but this study attempted to only describe communities in cabbage plants that already developed hearts (ca 25 weeks after sowing). Fungal communities change with the development of the plant, and the consensus is that the rate of infection increases with the host age [125]. Although the opposite trend was observed in early developmental stages of several *Brassicaceae* plants, where fast growing fungi increased in relative abundance and overall diversity of fungal and bacterial endophytes in fact decreased [13].

This study shows that a rich fungal community is associated with cabbages in later developmental stages in fields in New Zealand.

3.4.3 Conclusion

HTS is considered at most a semi-quantitative method due to several procedures and assumptions in the process of obtaining the final set of sequences [17, 133, 134].

Yet, in this study, important features describing the fungal communities associated with cabbage, such as abundance distribution and differences in composition

of the communities across plant tissue or regional scale remained very similar for both HTS and isolation through culture.

Nevertheless, it should not go unnoticed, that taxon assignment based on a reference database was somewhat problematic, and caused discrepancies in the two datasets in this study.

Furthermore, the chosen HTS methods clearly discriminated against fungi from the genus *Mucor* and the culture-dependent approach failed to recover any of the Chytridiomycetes.

It remains to be elucidated what the real biodiversity of fungi associated with cabbages is, since accumulation curves obtained by both methods suggest more species to be discovered.

Chapter 4

Can inoculation by fungi protect cabbage seedlings from *Sclerotinia sclerotiorum*?

4.1 Introduction

Sclerotinia sclerotiorum

Sclerotinia sclerotiorum is a fungal plant pathogen affecting hundreds of plant species in several families, including many important agricultural and horticultural crops such as vegetable brassicas, oilseed rape, lettuce, peas, sunflowers, beans and carrot [66, 65, 100, 110, 122].

Cabbage can be susceptible from the seedling stage through to a developed head and even during storage and transportation. Most of the infections in cabbage occur after the head formation and are thus referred to as head rot [65].

Stem rot caused by *Sclerotinia* in canola (*Brassica napus*) causes variable yield losses up to 100% in susceptible cultivars [117, 110]. It is a major fungal disease compromising yields of canola in countries around the world, including but not limited to Australia, China, United Kingdom, United States, Germany and South Africa [40, 70, 27, 117, 122]. The disease development depends on percentage of infection, variety and growth stage of the host plant. In New Zealand *Sclerotinia* causes an estimated $5-11 million loss for the kiwifruit industry per year through fruit scarring and petal blight [59].

Mechanisms of pathogenicity

Sclerotinia is a necrotrophic plant pathogen with a life cycle including a mycelial phase and a resting phase. *Sclerotinia* survives in soil as sclerotia which germinate as mycelium, to directly infect the crop, or as a fruiting body producing windborne ascospores which infect the above ground parts of the crop. *Sclerotinia* requires humid conditions to develop and produce the sclerotia or fruiting bodies [65, 117].

Sclerotinia uses a wide array of lytic enzymes including cellulases, hemicellulases, pectinases and acidic proteases to degrade host plant cell walls and disintegrate host plant tissue. High activity of these enzymes is ensured by an acidic

environmnent which results from the fungus excreting oxalic acid [117]. Not only does the oxalic acid activate the lytic enzymes, it also interferes with host plant cell signaling through calcium or oxidative burst [117]. Indeed, mutants of *Sclerotinia* unable to produce oxalic acid obtained in a study by Dickman et al. [28] were non-pathogenic. The affected tissue undergoes maceration which results in symptomatic water-soaked necrotic lesions sometimes with patches of white fluffy mycelium [117].

Control of *Sclerotinia*

Many strategies to control the disease caused by *Sclerotinia* were developed since its discovery in 1862. These include cultural practices, fungicides and biological control.

Preventing build up of sclerotia in the soil by growing non-susceptible intercrops may reduce the disease incidence. No-till farming may promote bacteria that colonise and degrade the sclerotia. Furthermore there is a relation between viability of the sclerotia and the depth of their burial [117].

Fungicides continue to be very effective in fighting the disease. The benzimidazole family of fungicides, such as carbendazim, pthalimide fungicides, such as captan or thiophanate, are widely used among others [117]. Chemical treatment can be very effective, as was shown with thiophanate and captan, where 100% of the fungi were eradicated from the infected seeds [117].

However, high disease pressure and difficulty in effective timing of fungicide application and penetration can allow the disease to establish [65]. Furthermore, fungicide resistance has been reported in *Sclerotinia* against benomyl, fludioxonil, captafoland or chlorothalonil [65, 97].

What is more, fungicide application is increasingly regarded as unsustainable and may be associated with many human health and environmental risks. For example many azole fungicides are endocrine disrupters – in other words they disrupt hormone production in mammals including humans [152]. Recently Lukowicz et al. [76] showed that a cocktail of commonly used pesticides including boscalid, captan, thiofanate and ziram worked synergistically to induce sexually dimorphic obesogenic and diabetogenic effects on mice. Chlorothalonil, a widely used fungicide, increases mortality of amphibians and acts as an endocrine disruptor at expected environmental concentration, to which humans are commonly exposed [80].

Biological control using bacteria or fungi is another approach to decrease *Sclerotinia* damage. Antagonism of some endophytic fungi towards *Sclerotinia* spp. was shown in several studies where different methods of action came to play. Rocha et al. [105] isolated four fungal endophytes from comfrey (*Symphytum officinale*) which showed consistent growth inhibition of *S. sclerotiorum* in dual cultures (both fungi co-cultivated in same petri dish). Zhang et al. [153] isolated 24 fungi from oilseed rape which significantly inhibited growth of the pathogen in dual cultures. Filtrates of broth cultures from isolates of four species (*Aspergillus flavipes*, *Chaetomium globosum*, *Clonostachys rosea* and *Leptosphaeria biglobosa*) showed suppression of leaf blight caused by *S. sclerotiorum*.

Jones et al. [65, 66] tested fungal isolates in soil that suppressed *Sclerotinia*

disease in cabbage and lettuce respectively. From the fungi used in these studies, *Trichoderma* spp., are known to colonise plants (including *Brassica* spp.) as endophytes [38, 103].

Many more fungal antagonists have been used to control *Sclerotinia in vivo* including *Alternaria*, *Fusarium* or *Ulocladium* [122]. *Ulocladium* not only suppressed growth of *Sclerotinia* in dual culutre but also prevented infection of leaves when applied on infected petals of canola [74]. *Alternaria* and *Fusarium* suppressed lesion formation on celery petals in growth-room and greenhouse trials but had inconsistent effects in field trials in a study by Inglis and Boland [61].

For many of the reasons listed above, farmers are increasingly looking for ways to control diseases in an environmentally friendly way. Use of biological control agents such as fungi is considered less toxic to humans and the environment than chemical pesticides [53, 19].

Aims of this study

This study aimed to evaluate the potential of fungi recovered from cabbage tissues (Chapter 2 and Chapter 3.2.1) as biological control agents against *Sclerotinia* in a bioassay on cabbage seedlings.

4.2 Materials and methods

Cabbage seeds were potted in trays with soil-based potting mix. Each seed was inoculated by spore suspension of the selected fungal isolate or mild detergent as a control. Eight days after planting, cabbage seedlings received four droplets of mycelial suspension of *S. sclerotiorum*. Diameters of necrotic lesions were measured four days after *S. sclerotiorum* inoculation.

4.2.1 Experimental treatment

Fungal isolates used in current bioassay were selected from the collection of isolates recevoered as described in Chapter 2 and 3. Only sporulating isolates were used. Preference was given to isolates from different regions if a species was recovered multiple times (Table 4.1). Fungal isolates were grown for 3-4 weeks on 1/4 strength potato-dextrose agar (PDA) in a growth chamber at 18°C, with 12h/12h – light/dark period. Spores of each isolate were collected with 0.01% Triton and filtered through sterile cotton. Spore concentration was approximately 10^7 spores/ml. Cabbage seeds (*Brassica oleracea* var. *capitata*) were sown in six well trays in a small greenhouse. Potting mix was prepared by mixing 4l of bark and 1l of pumice amended by 150 g of Osmocote Exact Standard 3-4M fertilizer (Everris International BV, Netherlands) and horticultural lime and Hydraflo (Everris Australia Pty Ltd) 50 g each. Water level was kept saturated so that relative humidity was $> 80\%$. Each seed received 70 μl of spore suspension while control plants received 70 μl of 0.01% Triton X 100.

Species	isolates
Alternaria alternata	m7Ss6, a3Ss11
Bulleromyces albus	a5S14
Chaetomium spirochaete	m3Ra1
Cladosporium angustisporum	m4Ls5
Cladosporium exasperatum	a2La51,h5S13, m4Lp5
Cladosporium fusiforme	a2La6
Clonostachys rosea	223S353
Fusarium acutatum	a3Rs1
Fusarium begoniae	a7La6
Fusarium boothii	m5Rs52
Fusarium circinatum	m5R122, a3L13
Fusarium concentricum	223R34
Fusarium equiseti	a7Sa3, m6S12
Fusarium sporotrichioides	112S2c2, 113S1d1, 212R2412, 222S162
Fusarium sublunatum	111R261, 111R263
Lecanicillium muscarium	h2La53, m2La6, a2R14, 223S3512, 223S352
Meyerozyma guilliermondii	a1Ss2
Monographella cucumerina	m3Ss32
Papiliotrema frias	h2L16
Penicillium aurantiogriseum	112S142
Penicillium brevicompactum	a1Lp51
Penicillium commune	h6Ls6
Penicillium decumbens	a8L12
Penicillium novae-zeelandiae	111S342
Penicillium simile	a1Rs2, a7La31
Penicillium thomii	h1Ss22
Penicillium westlingii	213R36
Periconia pseudobyssoides	a4Sa6
Phoma plurivora	m4Sa1
Purpureocillium lilacinum	m3R151, h1Rp5
Sporobolomyces roseus	m4Ss1
Stagonosporopsis dorenboschii	m8L15
Trichoderma atroviride	a1Rs1, a2Ss4, 111L34, 212S2c
Trichoderma piluliferum	h6S13, h6Sa1, h6Ss5
Ulocladium chartarum	m5La1, 112S261, 113S25, 114L231, h3Sp4, 114S35
Verticillium isaacii	m7Ss32
Wickerhamomyces anomalus	a1Lp2

Table 4.1: Fungal isolates evaluated for control of *Sclerotinia* in bioassays on cabbage seedlings.

4.2.2 Disease inoculation

S. sclerotiorum inoculum was prepared as a mycelial suspension. *S. sclerotiorum* (from the Linoln University Culture Collection – LU8806) was grown on 1/4 strength PDA in a growth chamber at 18°C, 12h/12h light/dark. Three plugs from this colony were transferred to liquid medium (100 ml of potato-dextrose broth) and cultivated on a shaker for 5 days at 120 rpm and room temperature. Eight days after planting, the cabbage seedlings were inoculated with *S. sclerotiorum* prepared by homogenising the liquid culture in a blender for 3 minutes and filtering through a kitchen stainless steel sieve. The concentration of mycelial suspension was 20 x 10^4 fragments/ml. Each seedling received a total of 4 droplets of mycelial suspension of 10 μl, one droplet on each cotyledon lobe (Figure 4.1). There were six bioassays in total, each testing a subset of the 52 isolates. Each bioassay was laid out in a randomised block design.

Figure 4.1: Cabbage seedlings inoculated with droplets of mycelial suspension of *S. sclerotiorum*.

4.2.3 Measurements

The diameters of necrotic lesions were measured 4 days after inoculation using a linear ruler (Figure 4.2). In case the lesion spread significantly over the cotyledon lobe and the diameter of the lesion could not have been properly measured, 10 mm was asigned as the highest possible value. Seedlings with 3 or more lobes only were used in evaluations. For statistical calculations datapoints were represented by average lesion diameter for each plant. ANOVA test and a post-hoc LSD test were performed to assess the differences between treatments in respective trials [99]. Seedling emergence was also recorded and evaluated using a Chi-square test [99].

Figure 4.2: Cotyledons of cabbage seedling with a necrotic lesion caused by *Sclerotinia sclerotiorum*. Seedling on the left displaying substantially larger lesions than the seedling on the right.

4.3 Results

None of the tested fungal isolates significantly affected lesion diameter caused by *Sclerotinia* compared to the control plants (Figures 4.3 – 4.5). However, there were significant differences in average lesions diameter between different experimental dates.

Seedling emergence was not affected by any of the isolates either. But again there were significant differences in emergence rates between different experimental dates.

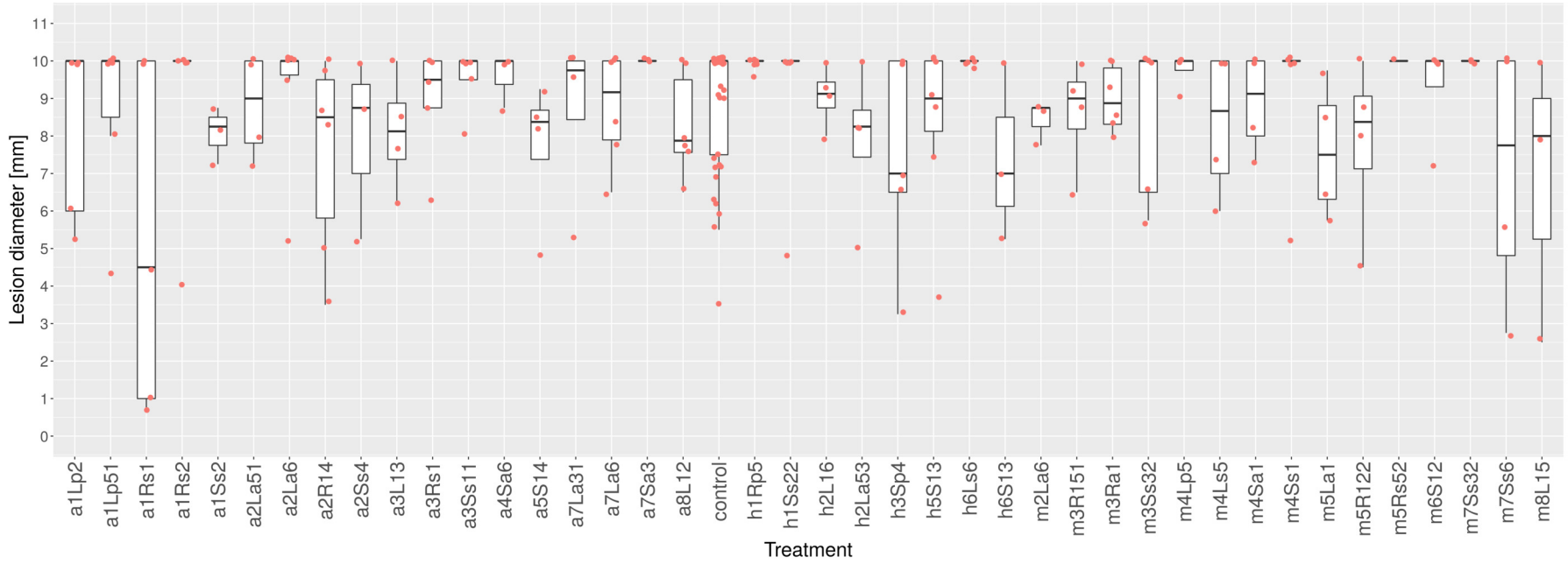


Figure 4.3: Boxplots of lesion diameters caused by *Sclerotinia* as measured on 8.7.2017. Boxes span the interquartile range (values between first and third quartile), the black segment in box represents the median, while whiskers show the largest/lowest value if not further than 1.5 x interquartile range from the box. Red dots represent mean lesion diameter of a plant in respective treatment.

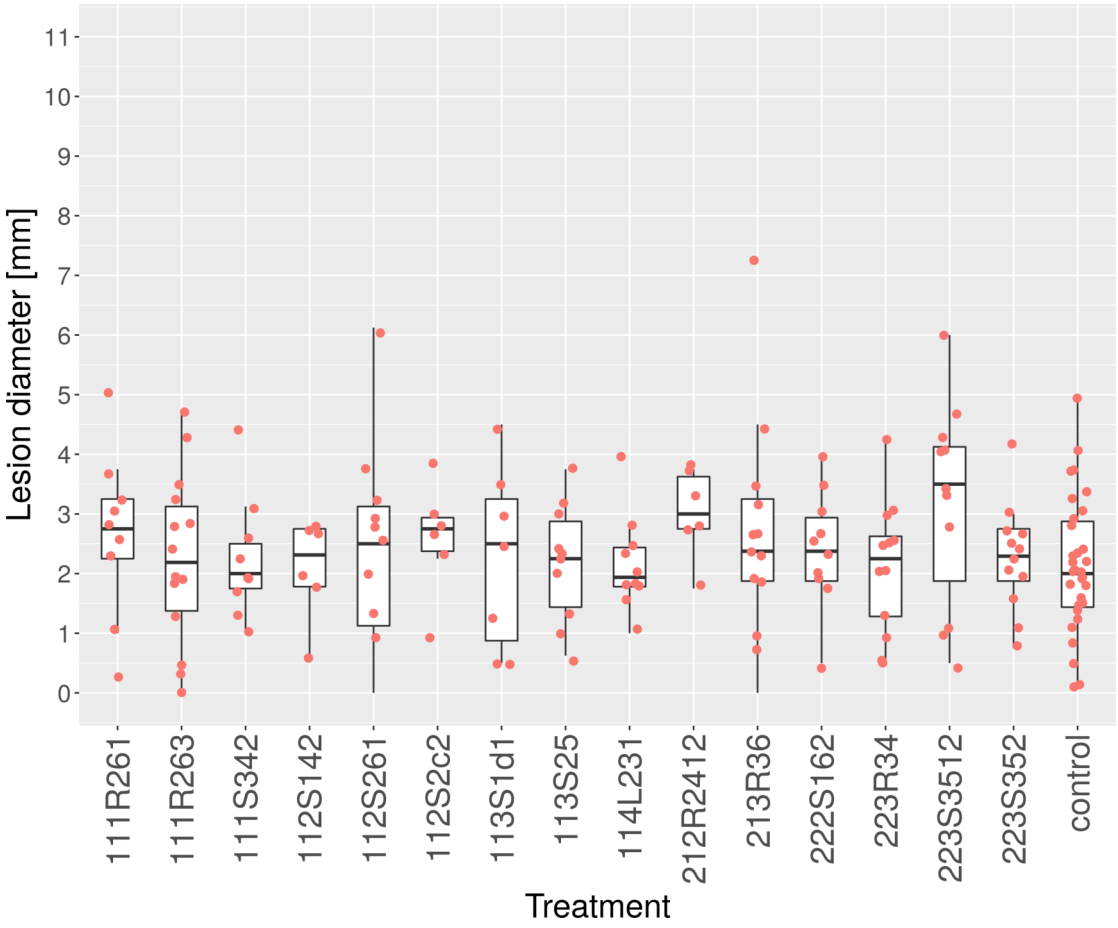
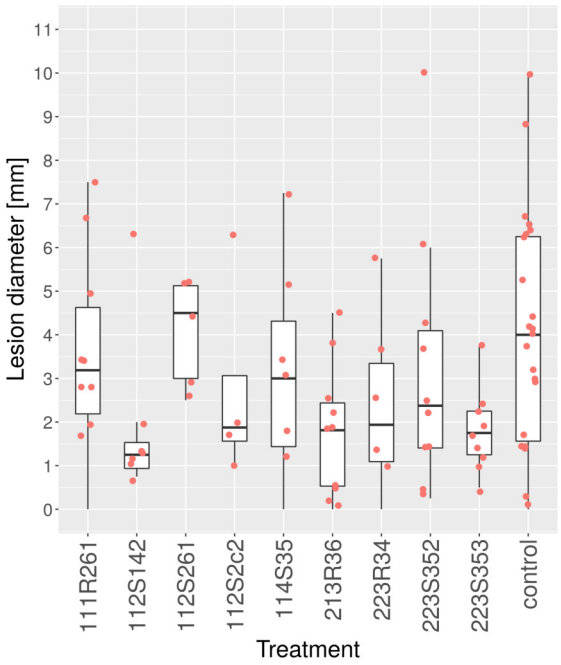
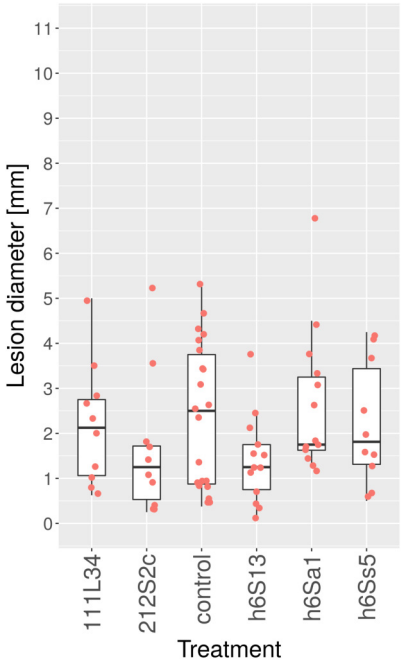


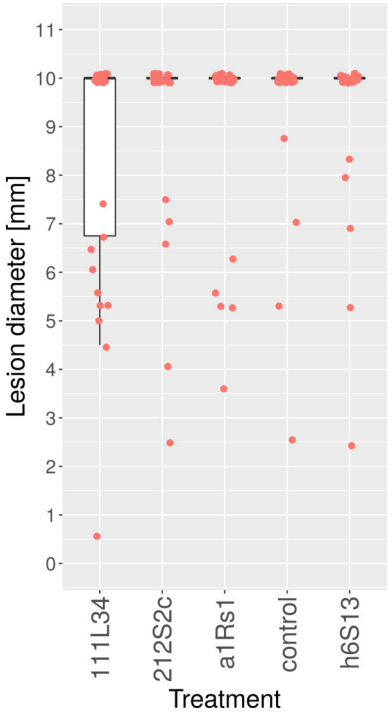
Figure 4.4: Boxplots of lesion diameters caused by *Sclerotinia* as measured on 26.10.2017. Boxes span the interquartile range (values between first and third quartile), the black segment in box represents the median, while whiskers show the largest/lowest value if not further than 1.5 x interquartile range from the box. Red dots represent mean lesion diameter of a plant in respective treatment.



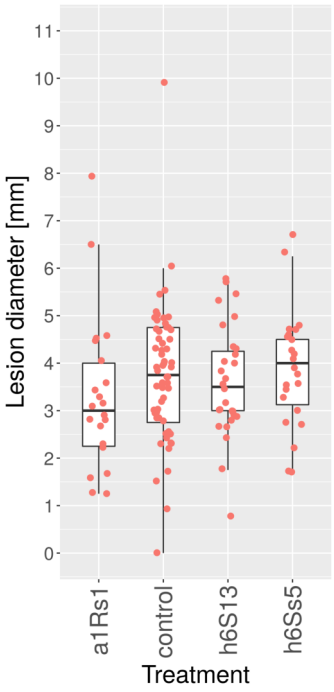
(a) Bioassay on 29.11.2017



(b) Bioassay on 11.1.2018



(c) Bioassay on 23.4.2018



(d) Bioassay on 9.5.2018

Figure 4.5: Boxplots of lesion diameters as measured by different treatments. Boxes span the interquartile range (values between first and third quartile), the black segment in box represents the median, while whiskers show the largest/lowest value if not further than 1.5 x interquartile range from the box. Red dots represent mean lesion diameter of a plant in respective treatment).

4.4 Discussion

The results of this study found no significant effects of selected fungal isolates on either emergence of cabbage seedlings or their susceptibility to *Sclerotinia* disease.

Even though fungal species related to some of the fungi tested in this study have suppressed *Sclerotinia* disease at some stage of cabbage development in other studies, none of the isolates used in this study have done so.

Jones et al. [65] have shown that *Trichoderma hamatum* and *Coniothyrium minitans* can suppress *Sclerotinia* infection of cabbages, possibly by parasitism and inducing systemic resistance. Other studies have reported fungi from genera *Alternaria*, *Fusarium*, or *Ulocladium* to suppress *Sclerotinia* disease development *in vitro* and *in vivo* [122].

The approach to test the fungi in this study dealt with the trade off between a high-throughput screening, such as dual culture, and in-field conditions, that would be the ultimate system where biological control agents would operate. Therefore, an *in vivo* approach with reasonable throughput was used in this study, similar to Garg et al. [40], where different genotypes of canola were challenged by droplets of mycelial suspension of *Sclerotinia* deposited on the cotyledons. The current study, instead of testing multiple cultivars, focused on a single cultivar of cabbage, treated with spore suspension of putative endophytic fungi upon sowing. This approach somewhat mimicked the one used in a study by Taylor et al. [131], where a seven week old plants were challenged by a mycelial plug of *Sclerotinia* on lower leaves.

Differences of the range of lesion diameters between experiments were likely caused by fluctuations in temperature or by different humidity levels in mini greenhouses. This was accounted for, by comparing treatments only within respective experiments.

No significant differences between lesion diameters in this study could have been caused by low effect size of the tested treatments in this experimental design. To overcome such limits, more repetitions and fewer experimental treatments were assessed in consecutive trials. More focus was given to *Trichoderma* strains in the later experiments due to extensive literature on the potential of *Trichoderma* to control *Sclerotinia*. However, even decreasing number of experimental treatments and increasing number of replications did not result in detecting a significant effect of tested strains.

The dynamics of the interaction between fungal isolates, cabbage and *Sclerotinia* could also have caused no effects to be detected. Some endophytic fungi, including various *Trichoderma* species, can induce plant host resistance by systemic immune response as was reviewed by Shoresh et al. [120]. However, fungal isolates in this study might not have had enough time to establish a reasonable association with the seedlings to induce systemic immune response.

Perhaps a different approach, where more time would be allowed to establish a possible endophytic or systemic immune response–inducing association between cabbage and the tested fungus, might have yielded different results. However this type of approach would not permit testing large numbers of fungal isolates due to time and equipment constraints.

Were it permitted by time and means in the project, a change in experimental

design might have detected smaller effects, which is, however, not always desirable for commercialisation. Nevertheless, a biological control agent even with a small effect could be applied in fields as a backup, or to work in synergy with other means of control, considering the need for diversification of strategies to control fungal pathogens.

Chapter 5

Pathogenicity of selected fungal isolates to Diamondback moth larvae

5.1 Introduction

Diamondback moth

The Diamondback moth (DBM) – *Plutella xylostella*, is an insect pest from the order Lepidoptera, family Plutelliadae. DBM is a prominent pest of plants of the genus *Brassica*, including vegetables such as cabbage, but can also attack forage brassicas and canola [37]. Worldwide vegetable *Brassica* production is estimated to contribute US$26 billion to the world economy. However the cost of DBM control combined with crop yield losses can reach US$2.7 billion annually [37].

Diamondback moth has four life stages – adult, egg, larva and pupa. The adult moths usually mate the same day they have emerged, and female moths lay eggs soon after. It takes five to six days for the larvae to emerge and the first instar larvae mine the leaves of brassicas. Subsequent larval stages usually consume the whole tissue of the leaf except for the waxy layer of the adaxial side. Around twenty days after emergence the fourth instar larva creates an open-network cocoon (Figure 5.1) and emerges into an adult one to two weeks afterwards. DBM adults are usually short-lived with a lifespan of several weeks, however there are indications that adults, larvae and pupae are able to withstand mild winters in temperate regions [130, 52].

Control of DBM

DBM control is currently done mostly through the use of broad-spectrum synthetic insecticides. However, indiscriminate use of insecticides have lead to insecticide resistance in various populations of DBM. There are more than 80 compounds falling into all major classes of insecticides, for which resistance in DBM has been recorded [37].

Insecticide application is increasingly regarded as unsustainable and may be associated with many health and environmental risks. Many of the widely used

Figure 5.1: DBM larva constructing an open network cocoon on a cabbage leaf.

pesticides are known to disrupt the endocrine system in humans; for example organochlorine pesticides were linked to developmental abnormalities, disruption in hormone levels, gestational diabetes and even fetal death related to birth defects [41]. Organophosphates such as chlorpyrifos are linked to neurodevelopmental delays, impaired gross and fine motor skills and behavioral problems [41]. On top of that Lukowicz et al. [76] showed that chlorpyrifos had a synergistic effect in a cocktail of commonly used pesticides where it induced obesogenic and diabetogenic effects on male mice.

Broad-scale insecticides may also be harmful to other insects, which could include natural predators of DBM, therefore reducing the environmental capacity of controlling DBM populations [37].

Cultural practices such as intercropping or trap cropping can reduce the impact of DBM on *Brassica* crops. Intercropping with non-host species such as tomatoes has been shown to reduce DBM populations or slow their life cycle. However there are also studies that show no significant effect [52]. Trap cropping is a practice similar to intercropping, where plants that concentrate immigration or oviposition are grown within, or on the edges of cash crop fields. The overall number of eggs laid on the field may in fact increase if the plants used in trap cropping are host plants (e.g. white mustard). An alternative approach is so called "dead end" trap cropping where the plants such as yellow rocket (*Barbarea vulgaris*) act as a sink by decreasing larval and pupal survival [52].

Furthermore, cultural practices such as polyculture with adjacent non-crop vegetation and reduced bare ground, can affect the diversity and strength of natural biological control agents [46, 116].

Biological control of DBM

DBM has a wide range of natural enemies including viruses, fungi, bacteria, generalist predators and parasitoids [52]. Parasitic wasps can attack larvae or pupae of DBM, the most popular ones in research and in the field are wasps of the genera *Diadegma*, *Diadromus*, or *Cotesia* since some of them can reduce DBM populations by up to 80% [52, 46].

The use of the bacterium (*Bacillus thuringiensis*) has proven to be very effective in many parts of the world. However, DBM was the first insect to develop field resistance to *Bt* toxin, probably due to indiscriminate use, similar to synthetic

insecticides [37].

DBM is susceptible to several genera of entomopathogenic fungi. Fungal genera such as *Lecanicillium*, *Isaria*, or *Metarhizium* have shown a promising ability to control DBM populations. Nevertheless, the most widely used in field conditions is *Beauveria bassiana* [46].

Biological control of DBM is a promising tool to tackle the damage caused by this insect, especially because of possible synergistic effects of different biological control agents as shown with *Beauveria* and *Bt* toxins or DBM predators attacking different larval stages [46].

Furthermore, farmers are increasingly looking for ways to control insect pests in an environmentally friendly way. Use of biological control agents is considered less toxic to humans and the environment than chemical pesticides [19, 53].

Aims of this study

This study aimed to evaluate fungi recovered from cabbage tissues in 2015 (Chapter 2) and 2016 (Chapter 3.2.1) for their potential to control DBM larvae in a bioassay on cabbage leaf discs.

5.2 Materials and methods

Second instar DBM larvae were fed leaf discs from commercially grown cabbage covered in spores from selected fungal isolates to test the insecticidal properties of these fungi.

5.2.1 Experimental treatment

Fungal isolates used in current bioassay were selected from the collection of isolates recevoered as described in Chapter 2 and 3. Only sporulating isolates were used. Preference was given to isolates from different regions if a species was recovered multiple times (Table 4.1). Fungal isolates were grown for 3-4 weeks on 1/4 strength potato-dextrose agar (PDA) in a growth chamber at 18°C, with 12h/12h – light/dark period. Spores of each isolate were collected with 0.01% Triton, filtered through sterile cotton. Spore concentration was approximately 10^7 spores/ml.

A disc was cut from the cabbage leaf using a 30 mm cork borer. Leaf discs were washed thoroughly in sterile distilled water and dried using tissue paper prior to treatment. A filter paper disc (30mm diameter) was placed in 35 ml clear graduated plastic portion cup and moistened with 100μl of sterile distilled water. Fifty μl of spore suspensions were deposited on each side of the leaf disc and spread by an inoculation loop so that the whole disc area was evenly covered in the spore suspension. Treated leaf discs were allowed to air dry for about 30 minutes before putting them on top of the moist filter paper disc.

Five second instar DBM larvae were placed in each cup with a leaf disc using a fine camel brush. The cup was closed with the lid and placed in an incubator at 25°C, with 12h/12h – light/dark period.

Species	isolates
Alternaria alternata	m7Ss6, a3Ss11
Chaetomium spirochaete	m3Ra1
Cladosporium angustisporum	m4Ls5
Cladosporium exasperatum	h5S13, m4Lp5
Cladosporium fusiforme	a2La6
Fusarium acutatum	a3Rs1
Fusarium begoniae	a7La6
Fusarium boothii	m5Rs52
Fusarium circinatum	h4Rs3, m5R122, a3L13, h4S111
Fusarium concentricum	223R34
Fusarium equiseti	a7Sa3
Fusarium sporotrichioides	112S2c2, 113S1d1, 212R2412, 222S162
Fusarium sublunatum	111R261, 111R263
Lecanicillium muscarium	h2La53, m2La6, **a2R14**, **223S3512**, **223S352**
Meyerozyma guilliermondii	a1Ss2
Monographella cucumerina	m3Ss32
Papiliotrema frias	h2L16
Penicillium aurantiogriseum	112S142
Penicillium brevicompactum	a1Lp51
Penicillium novae-zeelandiae	h6Sp2, 111S342
Penicillium simile	a7La31
Penicillium thomii	h1Ss22
Penicillium westlingii	213R36
Periconia pseudobyssoides	a4Sa6
Phoma plurivora	m4Sa1
Plectosphaerella oratosquillae	a7L13
Purpureocillium lilacinum	h1Rp5
Sarocladium kiliense	a5S112
Sarocladium strictum	m6S151
Sporobolomyces roseus	m4Ss1
Stagonosporopsis dorenboschii	m8L15
Trichoderma atroviride	a1Rs1
Trichoderma piluliferum	h6S13
Ulocladium chartarum	m5La1, 112S261, 113S25, 114L231
Wickerhamomyces anomalus	a1Lp2

Table 5.1: Isolates evaluated for control of DBM larvae in bioassays on cabbage leaf discs. Isolates that induced increased mortality are highlighted in bold font.

There were six bioassays in total, each testing a subset of the 52 isolates. Each bioassay was laid in a complete random design with 4 replications (Figure 5.2).

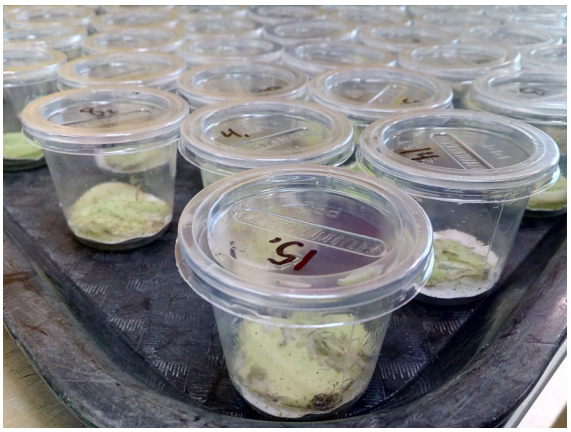


Figure 5.2: Cups in completely randomised design, each with 5 DBM larvae feeding on cabbage leaf disc placed on top of a wet filter paper disc.

5.2.2 Measurements

Larval mortality was assessed daily for at least 4 days after inoculation using a stereo microscope. Unless in a cocoon, live larvae were feeding and moving around the leaf disc and responded to even a slight shake or when touched by brush. Larvae were considered dead when not responding to physical stimuli, and when brown discolouring suggested decomposition of larval tissues (Figure 5.3).

Figure 5.3: A dead DBM larva, showing brown discolouring of internal tissues.

Mean mortalities were compared 4 days after inoculation in respective trials using an ANOVA test and subsequent Tukey's honest significant difference tests in "R" environment [99].

5.3 Results

Twenty of the 52 isolates tested failed to kill any DBM larvae. However, three isolates belonging to the genus *Lecanicillium* induced mortality greater than 80% and two isolates induced mortality lower than 50%. Isolate 223S3512 had the highest DBM mortality rate of 90% (Tukey HSD $p = 5 \times 10^{-11}$ Figure 5.7) followed by strains a2R14 and 223S352 both with 81.25% (Tukey HSD $p = 2.5 \times 10^{-4}$, Figure 5.6 and $p = 2 \times 10^{-9}$, Figure 5.7 respectively). *Lecanicillium* isolate m2La6 was also associated with increased mortality; in one experiment it caused 47.5% mortality which was significantly different from the control treatment ($p = 0.02$, Figure 5.4), however in a repeated experiment the mortality was only 43.8% and the effect was not significant in a post-hoc analysis (Figure 5.5c). *Lecanicillium* isolate h2La53 induced 25% mortality which statistically did not differ from the control (Figure 5.4). Results are summarised in Figures 5.4 – 5.7.

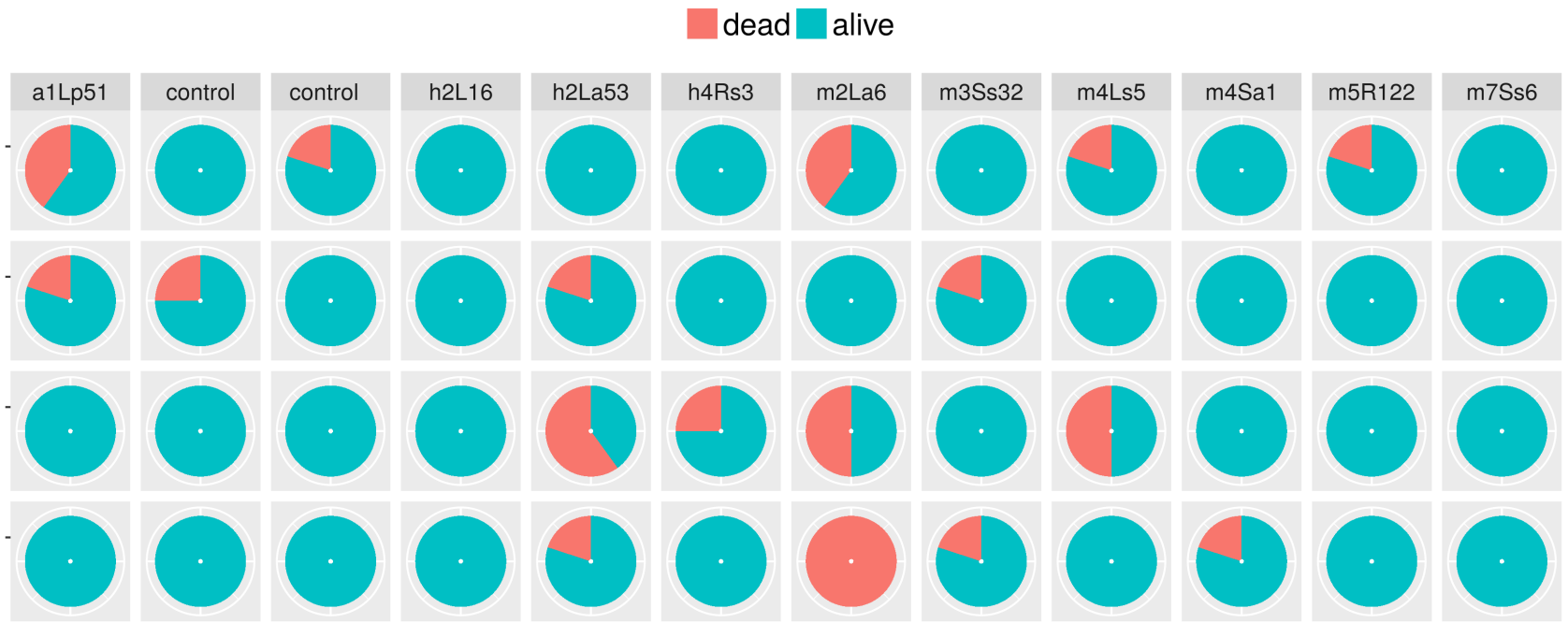
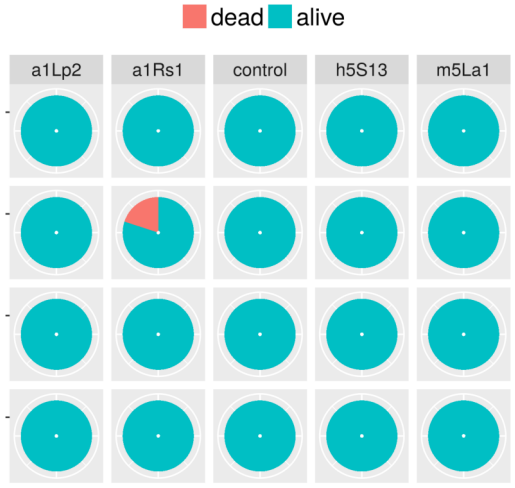
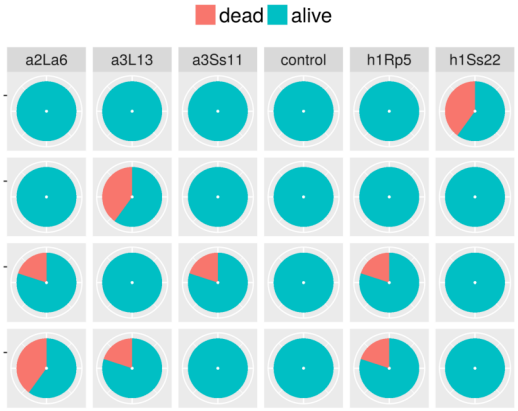


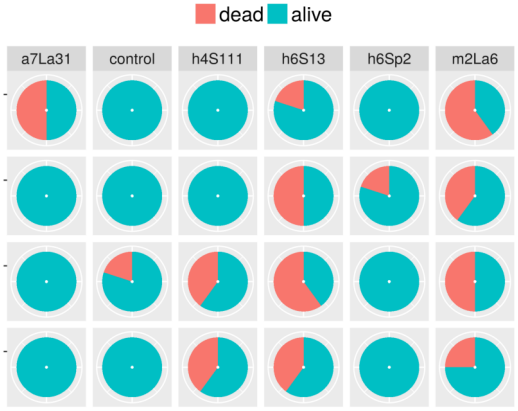
Figure 5.4: Mortality rates represented by fractions of living/dead DBM caterpillars in 4 replications of a treatment in the trial on 27.7.2017.



(a) 4.8.2017



(b) 10.8.2017



(c) 7.9.2017

Figure 5.5: Mortality rates represented by fractions of living/dead DBM caterpillars in 4 replications of a treatment in any given trial. Each trial is depicted in one graph labeled by the date when conducted.

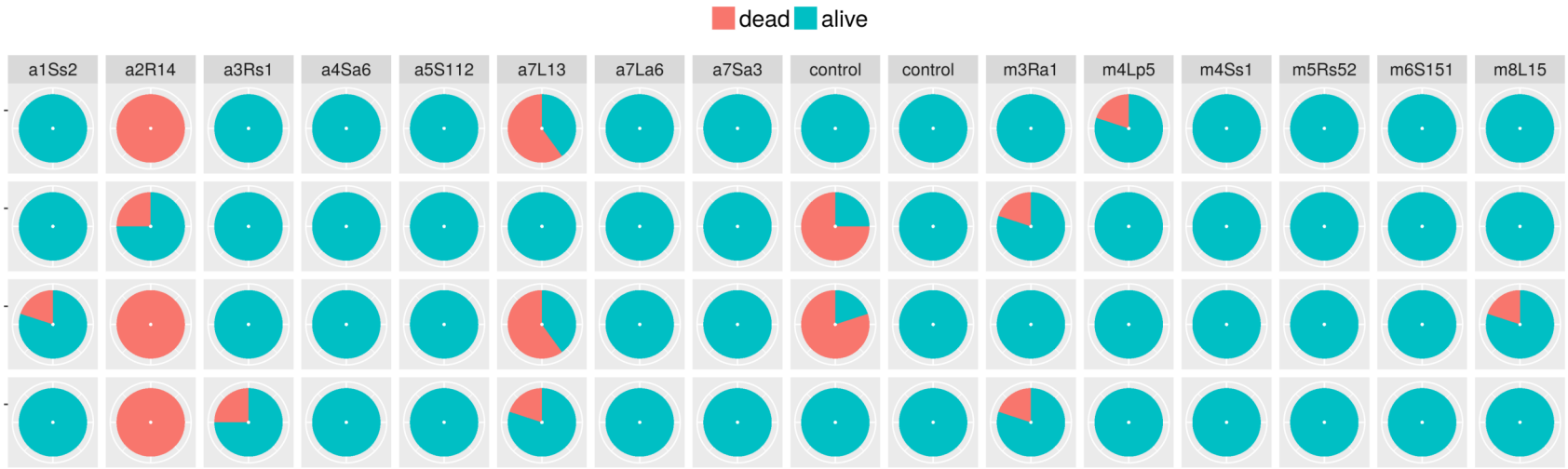


Figure 5.6: Mortality rates represented by fractions of living/dead DBM caterpillars in 4 replications of a treatment in the trial on 25.8.2017.

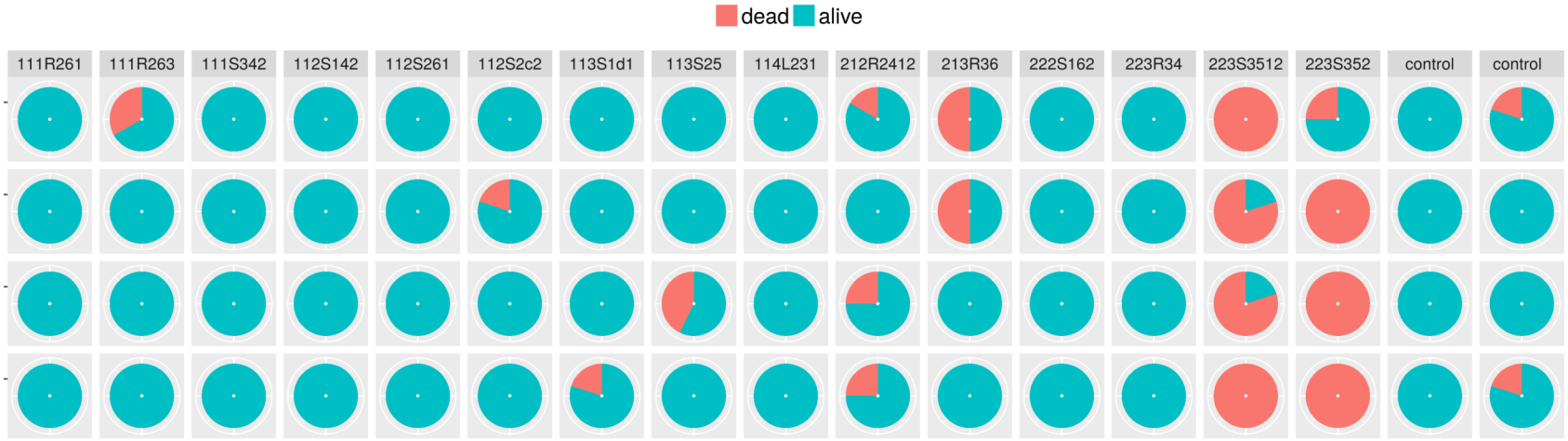


Figure 5.7: Mortality rates represented by fractions of living/dead DBM caterpillars in 4 replications of a treatment in the trial on 27.10.2017.

5.4 Discussion

This study found three fungal strains isolated from cabbage tissues, that increased mortality of Diamondback moth larvae in a feeding bioassay.

The pathogenicity of the *Lecanicillium* strains in this study is similar to results of other studies which reported the potential of *Lecanicillium* species as a biological control of DBM. However *Lecanicillium* used as a biopesticide rarely targets DBM; usually the controlled pests are aphids or nematodes [43]. Several commercial products based on *Lecanicillium* are summarised in a review by Brodeur [20] including products aimed to control aphids, mites, thrips and whiteflies. Of the reports showing potential for biological control of DBM, the effects of *Lecanicillium* were consistently better than no treatment, but were usually poorer than the most effective treatments, whether it was a synthetic insecticide or another entomopathogenic fungus. For example Duarte et al. [30] tested a *Lecanicillium muscarium* strain that caused more than 80% mortality of DBM larvae feeding on cabbage leaf discs, however fungal strains of *Beauveria* and *Metarhizium* were responsible for $98 - 100\%$ mortality. Yamada et al. [150] found that while their hybrid *Lecanicillium* spp. strains had killed half of the larvae after just two days, and resulted in more than 80% mortality in a screening bioassay, these isolates were less effective in field conditions.

In this study the DBM mortality ranged from 25% to 90% after 4 days as a result of contact with the *Lecanicillium* conidia. The variability of the pathogenicity could be attributed to genetic diversity of the strains. Bye and Charnley [21] showed that various strains of *Lecanicillium* possessed a distinctly expressed battery of cuticle-degrading enzymes suggesting different virulence strategies even between isolates of the same species. Similarly to the cuticle degrading enzymes, other metabolites involved in virulence may have differential expression, such as a recently described bassianolide which suppresses the immune response of DBM [68].

In a pursuit of biological control agents, screening many fungal isolates in laboratory conditions is only the first step. It needs to be followed by elucidating underlying mechanisms of action, if they are not yet known, and ultimately testing the limits of application and performance in field conditions.

Steps towards field application

If these strains were of interest for field application, more research would have to be conducted.

Ecology of the strains and their potential to endophytically colonise cabbage or other crops would also be of interest. This study only evaluated the effect of contact of DBM larvae with conidia on the surface of cabbage leaves, but there is evidence that endophytic fungi can influence insect feeding and development. For example *Acremonium alternatum*, a fungal endophyte, affected DBM feeding and growth rate when applied on roots of chinese cabbage [102]. Similarly endophytic *Metarhizium anisopliae* increased mortality of DBM larvae feeding on oilseed rape [14]. Furthermore, endophytic *Lecanicillium lecanii* in cotton plants slowed reproduction rates of aphids (*Aphis gossypii*) [48]. And since isolates in this study originated from internal plant tissues, there is a possibility they are able to endophytically

colonise plant tissues and perhaps could affect feeding and reproductive patterns or even mortality of DBM.

Potent *Lecanicillium* strains were isolated from stems (223S3512, 223S352) and roots (a2R14) of cabbage plants, while an isolate recovered from leaf tissue had barely a significant effect on mortality of DBM. Interestingly, the *Lecanicillium* isolate used to control aphids in a study by Gurulingappa et al. [48] originated from cotton leaves.

However, the tissue preference of the strains that increased mortality of DBM in this study is not yet known as well as their potential for DBM control when endophytic. Therefore research on the extent and nature of the interactions between the fungus and host plant could improve the biological control of DBM.

Before fungal biological control agents are used in large-scale field applications, there is a need to test their interactions with possible non-target species, especially the beneficial ones to prevent loss of ecosystem services [19, 109]. On top of that if the isolates are to be used on crops for human consumption such as cabbage, testing for human safety is imperative.

Conclusion

Three strains of *L. muscarium* showed potential as biological control agents of DBM, out of fifty two tested fungal isolates that were recovered from cabbage tissues. However, this study needs to be followed by further research if the fungal isolates are to be used as biological control agents in field conditions. Mechanisms underlying the observed variability of effects, temporal dynamics of the induced mortality as well as endophytic properties and tissue preference, and interactions with other organisms of these fungal isolates should be further investigated.

Chapter 6

General discussion

This work aimed to explore the ways of detecting endophytic fungal communities associated with cabbages, and the communities themselves. Isolation methods were optimised in an initial survey where a set of 68 fungal species was isolated from 10 cabbage plants. In the following year, cabbage plants from three geographical regions in New Zealand were sampled to isolate an additional 64 species and a culture-independent detection method was used to evaluate the non-culturable part of enodphytic fungal communities. Consequently, fungal isolates recovered from cabbage tissues were evaluated for their potential for biological control of a fungal plant pathogen *Sclerotinia* and the insect pest Diamondback moth (DBM).

6.1 Fungi associated with cabbage

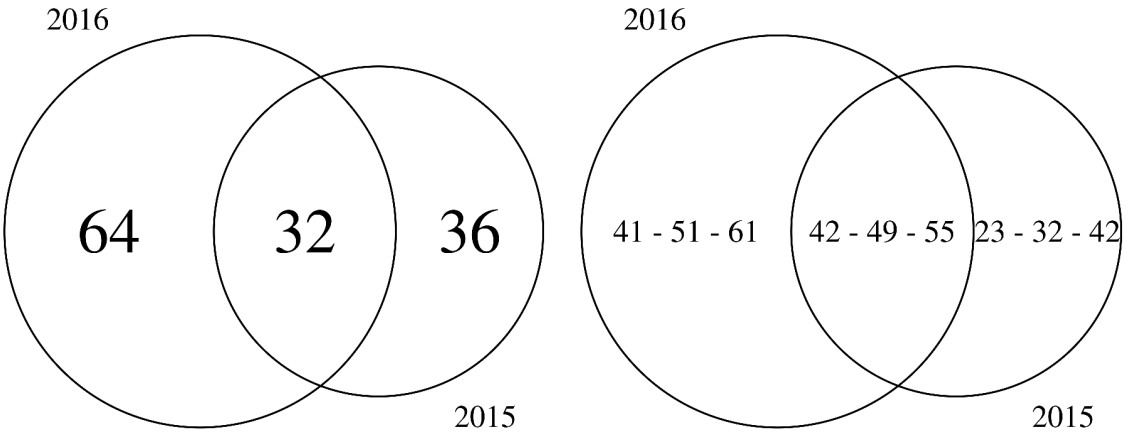
6.1.1 Isolated fungi

Overall 132 fungal species were isolated from cabbage tissues spanning over two years and three regions of sampling. The comparison of the respective sampling years shows that out of all 132 fungal species only 32 were detected in both years, nevertheless these represented 82% of all isolations (Figure 6.1).

Fewer species were recovered in both years than would be expected, nevertheless, these taxa stand out as ubiquitous and abundant; fungi such as *Alternaria*, *Boeremia*, *Cladosporium*, *Fusarium*, *Monographella*, *Plectosphaerella*, *Pyrenochaeta* and *Stemphylium* were isolated in most of the sampled plants and usually in high frequencies.

However, most of the species isolated in this study were rare. Such distribution of fungi is similar to that in a study describing mycorrhizal and phylosphere fungal communities [139], or to the one describing potential of endophytic fungi isolated from oilseed rape [153].

It is not possible to directly compare 2015 and 2016 studies, given differences in plant ages, isolation approaches, and sampled fields. Nonetheless, the relatively large number of species found in only one year or the other is consistent with a very high total diversity of fungi in these systems.



(a) Observed (b) Expected

Figure 6.1: Venn diagram showing distribution of species (a) observed and (b) expected described in Chapter 2 (2015) and Chapter 3 (2016). Expected numbers were based on random sampling of 999 iterations with 95% confidence intervals shown (lower confidence interval - mean - upper confidence interval).

6.1.2 Culture-independent detection

Culture-independent detection of fungi associated with cabbage in this study, and especially the comparison with the isolation dataset, is a relatively novel approach. To my best knowledge, no study has compared fungal communities in cabbage plants, except for a recent study focusing on development of fungal communities in cabbage and other plant seedlings by Barret et al. [13]. However, this study used only a sequence based approach.

As expected, the high-throughput sequencing encountered some limitations that are inherent to sequence-based methods. However, the resulting data on species distribution gave a similar picture of fungal species distributions to that obtained by culturing.

Several dominant taxa in both culture-dependent and independent datasets, that showed high sequence similarity were assigned different species names. This reveals a limitation that should be considered whenever interpreting the phylogeny of detected OTUs.

Nevertheless, species distribution in both the HTS and isolation datasets recognised similar spatial patterns of fungal species distribution showing distinct communities in different types of plant tissue and geographical regions. Furthermore, distribution of dominant fungal taxa corresponds in both detection methods.

The observed similarities and discrepancies suggest that culture-independent detection of fungi through HTS can be used to confidently describe distribution of OTUs, however the interpretation of the identity has its limitation, very likely due to the marker region used.

There is a growing number of studies evaluating culture-independent methods for detection of fungi, such as HTS. For example a study by Makiola et al. [77], where plant pathogens were investigated by HTS and cloning, showed that HTS is a reliable method to detect patterns of fungal communities. However, to my best knowledge, studies comparing fungal communities detected through isolation and culture-independent methods, such as HTS, are scarce and research in this study will hopefully add insights necessary to further adopt culture-independent techniques in describing fungal communities.

6.1.3 Shape of communities

The distribution of species in plant tissues and regions likely follows non-random patterns. Several species were isolated or detected in only certain types of tissue and the abundance of the species varied in different regions. Much stronger differences caused by tissue type could also reflect that the horticultural practices are similar in farms sampled.

The distribution of fungal endophytes in this study is similar to that described in a study of phyllosphere and mycorrhizal fungi by Unterseher et al. [139], where core species were abundant and detected in the majority of samples, whereas satellite species were found only in few samples and in low abundance. Such skewed distribution, however, is not limited to endophytic fungi and was reported for plant and animal communities in different spatial scales and seems to be common in most natural assemblages [142].

Distinct communities of fungi found in different tissues of cabbage aligns with non-random distribution of endophytic fungi described in several other studies [8, 107, 125, 146]. The data obtained in this study could serve as a guide or a reference for future bioprospecting of endophytic fungi in *Brassica* or other plants. Isolation targeting particular fungal species could focus on specific plant tissues thus reducing the amount of samples and associated costs. Furthermore our understanding of microbial communities and the networks of relationship is really at its beginning and this study adds an insight to a so far undescribed fungal communities associated with cabbage plants.

6.1.4 Beneficials or pathogens?

Many of the fungi isolated (and detected for that matter) are considered potential plant pathogens according to an online tool "FUNGuild" [84]. A table of isolated species and their expected trophic mode, updated synonymy based on NZFUNGI2 database provided by Manaaki Whenua Landcare Research [7], can be found in in a separate file deposited at Lincoln University Data Centre (Appendix E - Fungal guilds and synonymy of endophytic fungi isolated from cabbage in New Zealand 2015 and 2016. doi: 10.25400/lincolnuninz.9122507).

For example, species of the genus *Fusarium* are casual agents of various wilt diseases that also affect *Brassica* crops [69, 94]. However, pathogenic and non-pathogenic strains of *Fusarium oxysporum* were described in a study by Gordon and Martyn twenty years ago [44].

Several *Trichoderma* species are considered symbiotrophic endophytes, while others, such as *T. piluliferum*, display a range of trophic strategies including pathogenicity [84].

The "FUNGuild" database, however, classifies some fungi in a single guild; while other studies have shown different potential strategies of that taxon. For example *Clonostachys rosea* classified as a plant pathogen in "FUNGuild" was shown to enhance growth of cucumbers in a study by Sutton et al. [129].

These inconsistencies accentuate the volatile line separating enodphytes, saprotrophs and pathogens. It seems that many fungi found in plant tissues are, for the most part, harmless or do not cause visible disease symptoms. Besides, none of the 52 tested isolates showed any signs of pathogenicity towards cabbage seedlings, after they have been inoculated by spore suspension as described in Chapter 4. Furthermore, many studies have identified endophytic fungi to be useful in biological control against other pathogens or pests [22]. Eventually this might be the case for the current study where several *Lecanicillium* isolates induced increased mortality of DBM, an insect pest attacking *Brassica* crops.

6.2 Biological control is not a silver bullet

Although use of biological control agents is an approach increasingly sought after, it is not a cure for all problems associated with crop production. Rendering agriculture sustainable requires much more than biological control of pests [56].

Heavy reliance on fossil fuels and substantial amount of the environmental and health costs externalised need to be addressed on farms and on a worldwide scale [56]. However, this requires a strong political commitment and consumer awareness. Furthermore, farmers may be economically "locked" in unsustainable horticultural practice as explained in a study by Wilson and Tisdell [149]. Therefore, use of biological control agents alone simply cannot be enough to transition into sustainable agriculture. What it can be, though, is a useful tool in integrated pest management (IPM), where IPM is a holistic approach which should ultimately reduce pesticide use and economic costs while protecting environmental and human health [33]. It needs to be stressed, that the goal of IPM is not to discard synthetic pesticide use altogether, but rather to use it as a last, yet very effective tool to control pests. The inventory of tools implemented in IPM ranges from cultural practices such as crop rotation, no-till, conservation till farming or "pull push" crops, to the use of microbial antagonists and eventually use of synthetic pesticides.

This work does not aim to offer an exhaustive guide to control *Sclerotinia* or DBM, rather it aims to contribute to a battery of solutions needed to transition to sustainable agriculture through IPM. This study aspires to improve our understanding of agricultural systems, that is crops and associated microorganisms, and identifying potential biological control agents.

One of the aims of this study was to evaluate isolated fungal strains for their potential as biological control agents of *Sclerotinia* and DBM. Three *Lecanicillium* isolates showed promising results, as they increased mortality of DBM larvae feeding on cabbage leaves covered in their spores. However, this approach evaluated the

potential of biological control under specific conditions and the development of an efficient biological control agent requires much more research in order for these three isolates to be used in the field.

6.3 Future work

6.3.1 *Lecanicillium* as a biological control agent

Further insights into ecology of the isolates would be required not only to improve the efficiency and resilience of the biological control agents but also to ensure non-target effects are minimised, and that the application does not pose a risk to human health [57, 109]. Based on the study described in Chapter 5, the *Lecanicillium* isolates are likely to be used in inundative applications and as such would require further research into propagation, formulation and dosage and application manner and timing, with regards to the presence of susceptible pest stages [72].

The use of biological control agents, comes with the risk of the target pest developing resistance, similar to synthetic pesticides. A well described case of insect pests coevolving with their parasitoids, is described in a study from New Zealand by Tomasetto et al. [138]. It is likely that with the increase in use of microbial insecticides such as *Lecanicillium*, and especially indiscriminate use, targeted pests could develop resistance. Besides, certain DBM populations have already developed resistance to toxins derived from the entomopathogenic bacterium *Bacillus thuringiensis* [37].

Simultaneous use of several control agents is crucial in IPM, hence "integrated". In regard to synthetic pesticides, a study by Shaukat et al. [118] found that certain fungicides decreased growth, sporulation, conidial germination and cuticle-degrading enzyme production by *Lecanicillium muscarium*. However, another study showed that *Lecanicillium* could be used alongside several chemical insecticides [47]. Therefore, it seems that *Lecanicillium* has a potential as BCA in IPM, but care needs to be taken when combining control strategies.

It is important to prevent unwanted interactions of biological control agents with non-target organisms, in habitats where they are likely to perform. *Lecanicillium* species have a very broad host range including insects, mites, nematodes and fungi [20]. Therefore, specialisation of isolates from this study would have to be investigated further, if they were formulated as BCAs.

6.3.2 Potential for biological control of clubroot

This study aimed to explore fungi associated with cabbage and to identify potential biological control agents against DBM and *Sclerotinia*. However, during sampling for the study described in Chapter 3 in 2016, farmers expressed their concern with clubroot – a disease caused by the oomycete *Plasmodiophora brassicae*. Anecdotally, clubroot was hard to control and although crop rotation would reduce the incidence to some extent, the use of chemical fungicides could not be omitted. The clubroot disease is recognised to attack nearly all Brassicaceae species worldwide [22]. That is why, during the later stages of this project, a small study was designed to evaluate

the potential of selected fungal isolates obtained in this study to control clubroot disease in cabbage plants. The proposal and an outline of the study can be found in Appendix A - Biological control of clubroot disease in cabbage - a proposal.

6.3.3 Endophytes and *Brassica*

This study focused on the fungal component of the microbiome associated with cabbage plants in New Zealand. However, there is undoubtedly much more to the picture than just fungi, and perhaps the described patterns in fungal communities might not be complete since other microorganisms were deliberately ignored. In a review by Vandenkoornhuyse et al. [141], the authors use the term "holobiont" to stress that a plant and associated microorganisms are interdependent. The holobiont is defined as the host plant and associated microorganisms including but not limited to bacteria and fungi, protists or even nematodes.

Compared to many studies focusing on a group of organisms, such as in this study, there are relatively fewer studies investigating microbial communities across phylogenetic kingdoms [22].

There are at least twenty studies on bacteria and another twenty studies on fungi associated with Brassicaceae and their beneficial activity reviewed by Card et al. [22], however, none of these studies addressed fungi and bacteria simultaneously.

One of the few studies, that worked with a system somewhat closer to the concept of the holobiont, investigated the selection of bacteria and possibly fungi to alter traits in plant hosts including *Brassica rapa* and *Arabidopsis thaliana* (both from the Brasssicaceae family). Still, the authors only evaluated changes in the bacterial community [92]. A more holistic insight into the microbiome was given by Barret et al. [13], however, the authors focused only on seed and seedlings, and described the bacterial and fungal communities only through culture-independent methods.

Clearly the holistic approach lags behind the research focusing on a more narrow definition of the microbiome. The presented study, however, does not reduce this gap. Nevertheless, what this study aims to offer, is a deeper insight into fungal microbiome associated with cabbage plants as a reference for *Brassica* or other crops.

The current study focused on an agricultural crop from the family Brassicaceae, however, the associated cabbage relatives could also be of interest for the aims of this study.

Naturalised and new hybrids of brassicas and native plants form the Brassicaceae family can be found in New Zealand [51, 124, 83]. Growing in a vast range of environments, these plants could harbour microbial communities adapted to certain environmental conditions. Bioprospecting for beneficial endophytes in these environments could offer endophytes able to improve crop performance under specific stress factors [106, 92].

6.3.4 Conclusion

Endophytic fungi isolated from internal tissues of crop plants such as cabbage, have potential for improving crop production through biological control of pests. Diverse fungal communities can be detected, not only by isolation, but also culture-independent methods such as Illumina high-throughput sequencing. This study is far from being exhaustive in describing microbial communities associated with cabbage but gives a solid description of fungi associated with different plant tissues across different geographical regions. Three out of over fifty isolates tested, showed potential for biological control of Diamondback moth, however, further research needs to be conducted in order to formulate a biopesticide based on these isolates, In addition, more pests could be targeted by fungi isolated in this study.

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Appendices

Appendix A

Biological control of clubroot disease in cabbage - a proposal

A.1 Introduction

A.1.1 *Plasmodiophora brassicae*

Clubroot disease is caused by a soilborn plant pathogen *Plasmodiophora brassicae* that infects plants from the *Brassicaceae* family.

The clubbing prevents the roots from taking up water and nutrition and causes poor growth of the host plant and a severe reduction of crop production.

The life cycle of *P. brassicae* involves resting spores in soil, that germinate to zoospores which attack root hairs, and grow into multi-nucleate plasmodia (primary plasmodia) in the root hairs. The plasmodia cleave and then form secondary zoospores. The zoospores migrate to root cortical tissue and induce abnormal growth of the root tissue, forming a distorted massive gall, called a club.

The secondary plasmodia are formed in the clubs, then after meiosis, numerous resting spores are formed. Upon the decay of the clubs, resting spores are released into the soil, where they can survive for many years [55].

Increase in infections has been reported worldwide in the last few years where clubroot affects crops such as canola in Czech republic [143] or Canada [95], chinese cabbage in China [154] or cabbage in Japan [55], cabbage, broccoli or cauliflower in Australia [29]

New Zealand is not an exception and there has been already research trying to find ways to control the pathogen since at least 1960 when Lammerink (1964) [73] trialed dose response of clubroot inoculum on turnip, swede and canola.

Since then a lot of work has been done to come up with a possible treatment including but not restricted to control of soil pH, agrochemicals and biological control agents.

A.1.2 Possible treatments for clubroot disease

Control of soil pH has been reported as an effective strategy to control clubroot, since the infection occurs in acidic pH and alkaline pH along with prevents primary infections [81]. This effect can be achieved by applying mineral calcium or magnesium or organic matter rich in calcium [85].

Fungicides are widely used as a strategy to control clubroot, they usually inhibit germination of the resting spores. Benomyl has been used successfully for decades but more substances are being investigated. For example fluazinam was reported to increase yields up to 80% in a field trial in Australia when applied in bands along rows of transplanted broccoli and cauliflower [29].

The problem with fungicides is that they can adversely affect the soil biota and are not applicable in organic farming. Therefore a lot of research is oriented towards biological control agents (BCA) which are organisms capable of suppressing the disease.

Biological control agents

Bacteria applied as soil drench *Bacillus subtilis*, *Streptomyces lydicus* and *Streptomyces griseoviridis* all suppressed clubroot formation under different conditions in field or greenhouse trials on canola in Canada [95].

Fungi have also been investigated as possible biological control agents of *P. brassicae*. Commercially available formulation of *Trichoderma koningiopsis* reduced the disease incidence and severity and improved growth of cabbage in trials in Colombia [18] *Gliocladium catenulatum* applied as a soil drench reduced clubroot formation on canola in Canada up to the effect of commercially available fungicides [95]. Furthermore *Heteroconium chaetospira* was reported to suppress the club formation almost completely in sterile soil, effectively suppress it in non sterile soil, and even promote growth of chinese cabbage after extensively colonizing the root surface and its inner cortical tissues [82].

A.2 Aim of this project

The aim of this project is to test selected putative endophytic fungi isolated from cabbage plants in New Zealand for their potential to control clubroot formation on cabbage plants grown in greenhouse conditions. The possible mechanisms could include but are not limited to antimicrobial substances released as reported in or colonizing roots and preventing the disease to establish or priming the host plant immune system [82]. However, due to the length of this project the mechanisms of action will not be investigated.

A.3 Material and methods

A.3.1 Material

Clubroot infected soil from Plant and Food Research (personal comm. Mary Christey) will be used in the pot trials. Seeds of clubroot susceptible cultivars of cabbage (personal comm. Mary Christey) will be used in the pot trials. Up to 10 isolates of putative endophytic fungi including several *Trichoderma* strains, *Beauveria bassiana* and few others will be used in the pot trial. The isolates will be grown on Potato Dextrose agar (PDA) until sporulation is apparent (usually 2 weeks after inoculation). Spores will be extracted by washing the fungal colony in a Petri dish with approximately 5 ml of 0.1% Triton X and filtering through cotton bud. Spore concentration will be evaluated by haemocytometer.

A.3.2 Methods

Three seeds of the susceptible cabbage will be sown in each 1l pots filled with infective soils, drenched in 2ml of spore suspension (at 10^7 spores ml^{-1}) of respective fungal treatments prepared at the same day. Control plants will be treated with medium used for spore suspensions (0.1% Triton X). Each treatment will be replicated 20 times and control plants will consist of 60 replicates. The 240 pots will be completely randomized in a growth chamber. Plants will be grown for up to 8 weeks under $23\,^{\circ}\mathrm{C} \pm 1\,^{\circ}\mathrm{C}$, 16 h light for long day and 8 h light. One week after sowing each pot will be thinned so that only 1 viable seedling is present.

A.4 Expected outcomes

Disease symptoms will be assessed 8 weeks after sowing, using the disease index of Narisawa et al. (1998) [82]. Clubroot severity will be visually separated into six classes (0 = healthy root systems to 5 = completely clubbed) as reported in Cheah et al. [26] Fresh and dry weights and other growth characteristics such as number of leaves, or chlorophyll content might be assessed, to evaluate the effect of the treatments.

A.5 Timeframe

Expected start is late January 2018 when literature review, and cultivation of selected fungal isolates for the treatment will begin. Destructive evaluation of the experiment should take place late March with the data analysis and report completed by mid April 2018.

Appendix B

R scripts

Submitted as a seperate file at Lincoln University Data Centre with a doi: 10.25400/lincolnuninz.9122549.

Appendix C

Endophytic fungi from cabbage in New Zealand 2015

Submitted as a seperate file at Lincoln University Data Centre with a doi: 10.25400/lincolnuninz.9122513.

Appendix D

Endophytic fungi from cabbage in New Zealand 2016

Submitted as a seperate file at Lincoln University Data Centre with a doi: 10.25400/lincolnuninz.9122504.

Appendix E

Fungal guilds and synonymy of endophytic fungi isolated from cabbage in New Zealand 2015 and 2016

Submitted as a seperate file at Lincoln University Data Centre with a doi: 10.25400/lincolnuninz.9122507.